“A STUDY OF RELATIONSHIP OF ANTIMULLERIAN HORMONE IN WOMEN WITH NORMOOVULATORY AND POLY CYSTIC OVARIAN SYNDROME”

A DISSERTATION SUBMITTED FOR

M.D DEGREE BRANCH-XIII

[BIOCHEMISTRY]

DEPARTMENT OF BIOCHEMISTRY

CHENNAI MEDICAL COLLEGE HOSPITAL

AND RESEARCH CENTRE

IRUNGALUR

TRICHY

THE TAMILNADU DR.MGR MEDICAL UNIVERSITY

CHENNAI

APRIL-2016

CERTIFICATE

This is to certify that this dissertation entitled "**A STUDY OF RELATIONSHIP OF ANTIMULLERIAN HORMONE IN WOMEN WITH NORMOOVULATORY AND POLY CYSTIC OVARIAN SYNDROME"** is a bonafide record of the work done by **Dr.N.Naveetha Lakshmi,** under my guidance and supervision in the Department of Biochemistry Chennai medical college hospital and research centre ,irungalur,Trichy during the period of her postgraduate study form 2013 to 2016

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The work done by **Dr. N. NAVEETHA LAKSHMI** on titled "**A STUDY OF RELATIONSHIP OF ANTIMULLERIAN HORMONE IN WOMEN WITH NORMOOVULATORY AND POLY CYSTIC OVARIAN SYNDROME"** is under my supervision and I assure that this candidate has abide by the rules of the Ethical Committee.

GUIDE: **Prof.Dr.Kalavathy Ponniraivan.M.D.,**

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DECLARATION

I, Dr.N.Naveetha lakshmi hereby solemnly declare that the dissertation title "**A STUDY OF RELATIONSHIP OF ANTIMULLERIAN HORMONE IN WOMEN WITH NORMOOVULATORY AND POLY CYSTIC OVARIAN SYNDROME"** was done by me at Chennai Medical College Hospital And Research Centre, Irungalur, Trichy. Under the supervision and guidance of my Professor and Head of the Department **Dr.Kalavathy ponniraivan .M.D.,** this dissertation is submitted to Tamil Nadu M.G.R Medical University, towards partial fulfilment required for the award of M.D. Degree (Branch –XIII) in Biochemistry.

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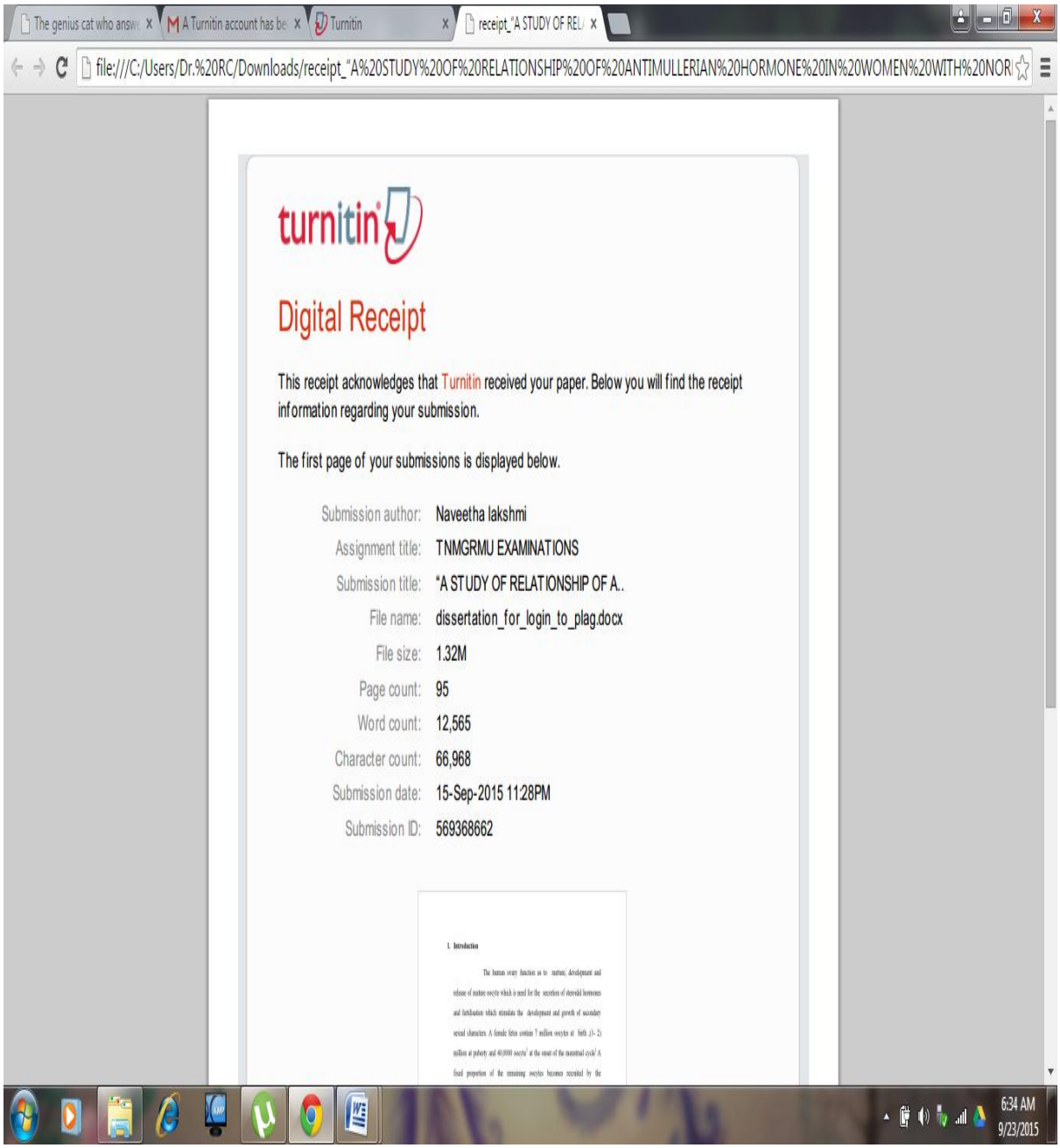

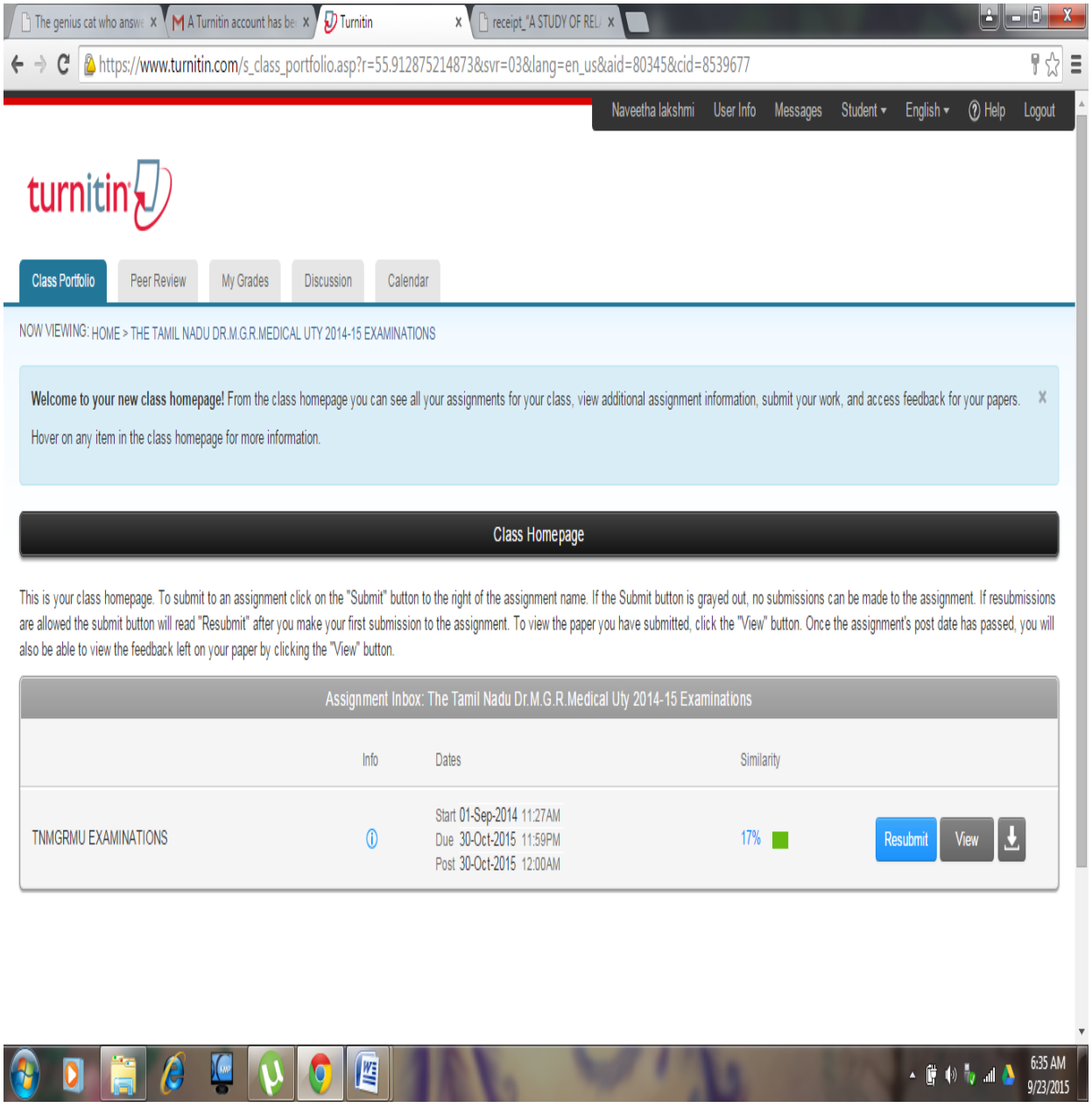
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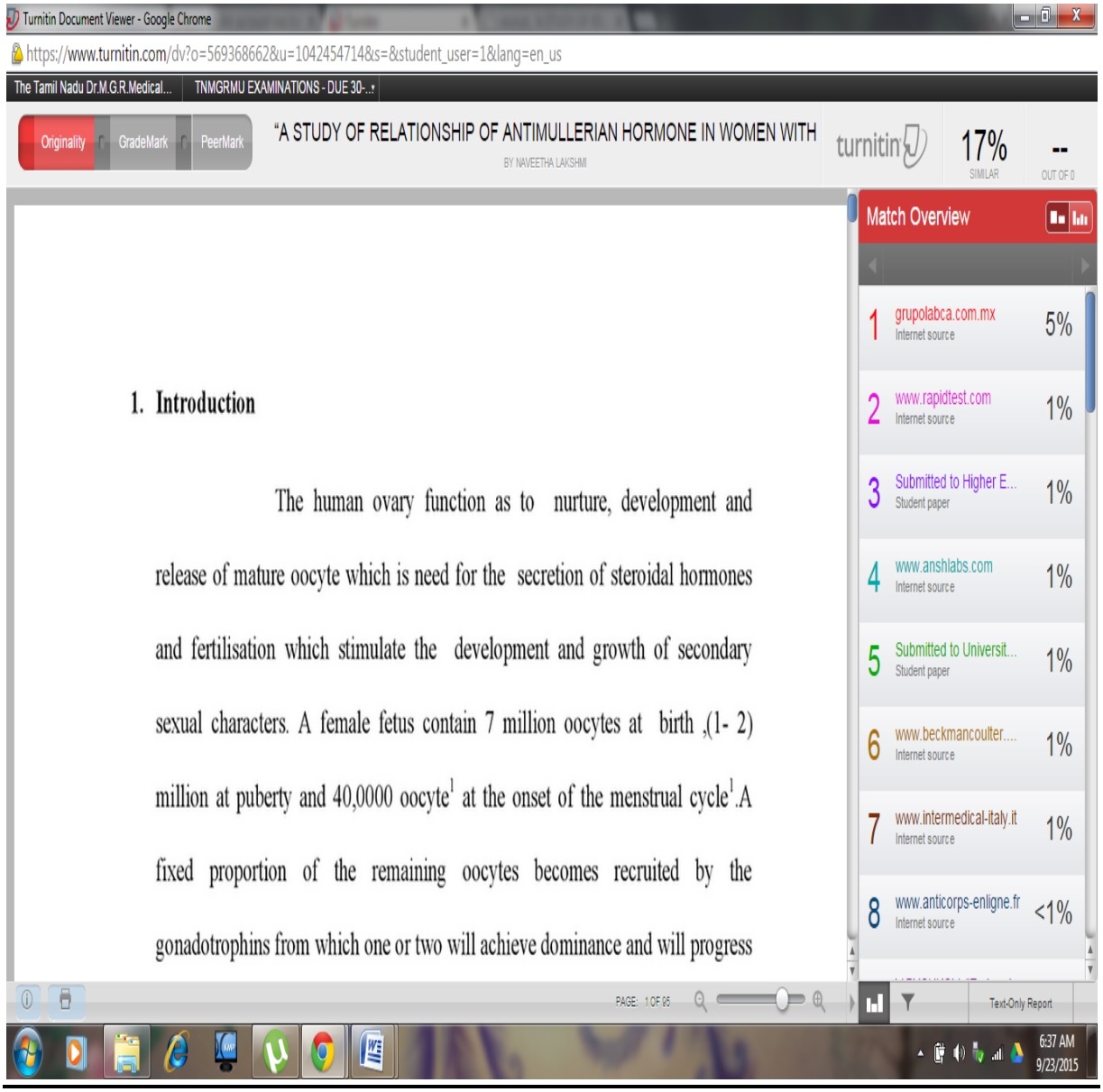
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The human ovary function as to nurture, development and release of mature oocyte which is need for the secretion of steroidal hormones and fertilisation which stimulate the development and growth of secondary sexual characters. A female fetus contain 7 million oocytes at birth ,(1- 2) million at puberty and 40,0000 oocyte[1] at the onset of the menstrual cycle[1].A fixed proportion of the remaining oocytes becomes recruited by the gonadotrophins from which one or two will achieve dominance and will progress
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to the ALMIGHTY for the

Successful completion of my study

CONTENTS

ABSTRACT

BACKGROUND:

Poly cystic ovarian syndrome is a heterogenous collection of signs and symptoms together form a spectrum of disorder where the clinical manifestation varies from mild to severe disturbances of reproductive endocrine and metabolic function. The pathophysiology of PCOS appear to be multifactoial and polygenic. AMH is a homodimeric glycoprotein which belongs to transforming growth factor β family. It is expressed in granulosa cells of secondary , preantral and small follicles ≤ 4 mm in diameter.

OBJECTIVE:

To estimate the levels of AMH in PCOS patients and Normoovulatory women and to correlate the same in both the groups.

MATERIALS & METHODS

This study was conducted in Chennai Medical College Hospital And Research Centre, Irungalur, Trichy.Thirty normal menstruating women and thirty women with anovulatory pcos with spontaneous or induced progesterone cycle were included in the study. Serum samples were collected and analysed for AMH and ultrasonographic measurement of AFC and ovarian volume were done in all subjects.

RESULTS:

The mean value of AMH among cases (11.28 ± 5.29) and controls (3.5 ± 1.46) were statistically significant by student's t test (p= < 0.001). AMH values in both groups were negatively correlated with age and positively correlated with antral follicular count. Furthermore the ovarian volume in cases were positively correlated with AMH and was statistically significant.

CONCLUSION:

The present study demonstrated that in PCOS serum AMH levels increased 2 to 3 fold higher than in control women. The raised AMH gives us the clue for the mechanism behind the anovulation in PCOS. AMH levels have a major inhibitory role during folliculogenesis thus leading to anovulation in PCOS.

ABREVATION

AFC	Antral follicle count
AMH	Antimullerian hormone
AMHR	Antimullerian hormone receptor
ART	Assisted reproductive technology
COH	Controlled ovarian hyperstimulation
E2	Estradiol hormone
ELISA	Enzyme linked immunosorbent assay
FSH	Follicle-stimulating hormone
GIFT	Gamete intrafallopian tube transfer
GnRH	Gonadotropin-releasing hormone
IVF	In vitro fertilization
LH	Luteinizing hormone
MIS	Mullerian-inhibiting substance
PRL	Prolactin hormone
T3	Triiodothyronine
T4	Thyroxine
TSH	Thyroid-stimulating hormone
TVUS	Trans vaginal ultrasound
OV	Ovarian volume

Introduction

The human ovary function as to nurture, development and release of mature oocyte which is need for the secretion of steroidal hormones and fertilisation which stimulate the development and growth of secondary sexual characters. A female fetus contain 7 million oocytes at birth ,(1- 2) million at puberty and 40,0000 oocyte[1] at the onset of the menstrual cycle[1].A fixed proportion of the remaining oocytes becomes recruited by the gonadotrophins from which one or two will achieve dominance and will progress to ovulation.

The quality and quantity of oocytes is determined by the term ovarian reserve[2].The reproductive potential is important and is measured by ovarian reserve [4].Ovarian reserve decline with age and the variations is significant in individual with the onset of decline in age and the assessment of the ovarian reserve is needed[3].

Measurement of ovarian reserve in disease like Polycystic Ovarian Disease aid in mode of selection of mode of treatment. The clinical features of PCOS is a spectrum of disorder with a heterogeneous collection of signs and symptoms from mild to serve disturbance of metabolic functions and Reproductive Endocrine. The Polycystic Ovarian Syndrome pathophysiology appears to be polygenic and multifactorial. The most common endocrine disorder is PCOS of the Reproductive age group women. Polycystic Ovarian Syndrome is diagnosed based on the presence of any two of the following three criteria according to Rotterdams (2003)[5],They are

i) Oligo and Anovulation –menstrual cycle longer than 35 days or less than eight menstruations periods per year.

ii) Hyperandrogenism –(clinical/biochemical) -as per the Ferrimann-Gallway scoring system designed to assess the clinical manifestation of Hirsutism[6].As per the scoring system the Masculine part of body hair growth is described in four degrees on different body places Viz. Upper lip ,Chin ,Chest ,Lower leg ,Upper back ,Upper abdomen ,Lower back, Arm, , ,Lower abdomen ,Forearm and Thighs.Biochemical manifestations is Hyperandrogenism which is calculated by Free androgen Index (FAI) from testosterone and sex hormone binding globulin(SHBG) [7,8].

ii) Polycystic Ovaries- Where at least one of the ovaries should have follicles measuring 2-9 mm in diameter or should be of 12 follicles (or) an increase in Ovarian volume (>10cm^3).[9]

A wide variety of heterogenisity of signs and symptoms exist in PCOS women and in some PCOS subjects who can exist without any clinical signs and symptoms which can be expressed over time.

PCOS is prevalent in young Reproductive age group where the distribution 20-30%[10,11]. Presence of Insulin Resistance, Dyslipidemia and Central obesity which might lead to the complications of Diabetes and Cardiovascular disease in PCOS women. Women with PCOS have been under the increased risk for Endometrial, Breast, and Ovarian cancer.

The functional potential of the Ovary reflects the number and activity of the oocytes present within the Ovary which refers to ovarian reserve. The ovarian reserve constitutes the oocytes and the ovarian follicular pool size that it diminishes with

increasing age. Ovarian reserve can be assessed by various markers Viz .serum FSH, serum Estradiol , Ovarian volume , antral follicle count etc.,

Ovarian reserve are assessed by Biochemical parameter and ultrasound parameter. Biochemical parameters are measurement of Hormones such as FSH and E_2 .Measurement Of FSH and $E_{2\ on}$ Day3[12] has limitations in PCOS patients as it is very difficult to predict the appropriate time owing to irregular cycles. Antral follicular count by ultra sonogram is a better marker for assessing the Ovarian reserve .The characteristic of the Polycystic Ovary are variable and may be subtle and need lot of expertise to precisely determine the AFC count. Owing to the above said limitations there is a need of a marker which correlates well with ovarian reserve.So as to ascertain the functional ability of Ovary and intervention of ovarian pathology for conception.AMH level decreases steadily with increasing age from 24 to 50 years of age[13].Reports documented AMH as a predictor of age related reductions in fecundability in the general populations.

AMH concentration in the serum is directly related to the antral follicle count and is a better indicator of Ovarian reserve when compared to FSH and Estradiol level[14].As the concentrations of AMH is unaffected by gonadotrophins it is feasible to measure AMH throughout the cycle[15].With this background, the present study aims at measuring AMH levels in normoovulatory and Polycystic Ovarian Syndrome to assess the Ovarian reserve.

There are few studies done in correlation of AMH with ultrasound findings ,hormonal profiles in normoovulatory/PCOS.The present study elucidates the

correlation of AMH in normoovulatory and PCOS subjects related to Biochemical (hormonal)and ultrasonographic findings .This will aid us to include measurement of AMH in PCOS subjects in diagnosing and for better management .

Aim of the study

To Study the relationship of Antimullerian hormone among Normoovulatory and Poly Cystic Ovarian Syndrome women.

Objectives of the study:

1. To study the level of AMH in PCOS patients
2. To study of the level of AMH in normoovulatory women.
3. To determine correlation of AMH levels in normoovulatory women and PCOS women .

Ovary

Ovary undergoes through a number of stages as it passes from the stage of development. The development is initiated at 30 days after the post conception by formation of a genital ridge, followed at about 35 days by an indifferent gonad, at 42 days developed into an embryonic ovary (6 weeks), and at about 8weeks an early fetal ovary is developed , at 16 weeks a late fetal ovary, and perinatal ovary develops by the end of 28 weeks. Functional ovary develops from the germ cells. The sex gonadal of an embryo becomes evident at about 6 weeks post conception,

Fetal early weeks span for about 8- 16 weeks. Primordial follicles are formed by the 12^{th} week ,when the epithelial cells surround ova and after 16 weeks of gestation they form the late fetal stage. The cuboidal epithelial cells emerge from the growing follicle by 28^{th} weeks of gestation .The stroma later differentiate into theca interna which demonstrates features of steroidogenesis, the presence of 3β-hydroxysteroid dehydrogenase [16]

Paired gonadal structures the ovaries which lie suspended between the pelvic wall and the uterus by the infundibulopelvic and uteroovarian ligaments. Adult ovaries are ovoid in shape and they measuring 5 X 3 X 3 cm.There is variation in dimension of the ovary in respect to hormonal changes during the menstrual cycle and also along with age. Normally small corpus luteum along with small cystic follicles are seen under the surface of the ovary. Each ovary consists of outer cortex composed of specialized stroma and follicles of various sizes, the inner

medulla occupies a small portion of ovary made of fibromuscular tissue and blood vessels.

Ovarian function

The ovaries have two functions: the production of ova and the production of hormones .Both these functions are controlled through the hypothalamic-pituitary ovarian axis by endocrine, paracrine and autocrine pathways.

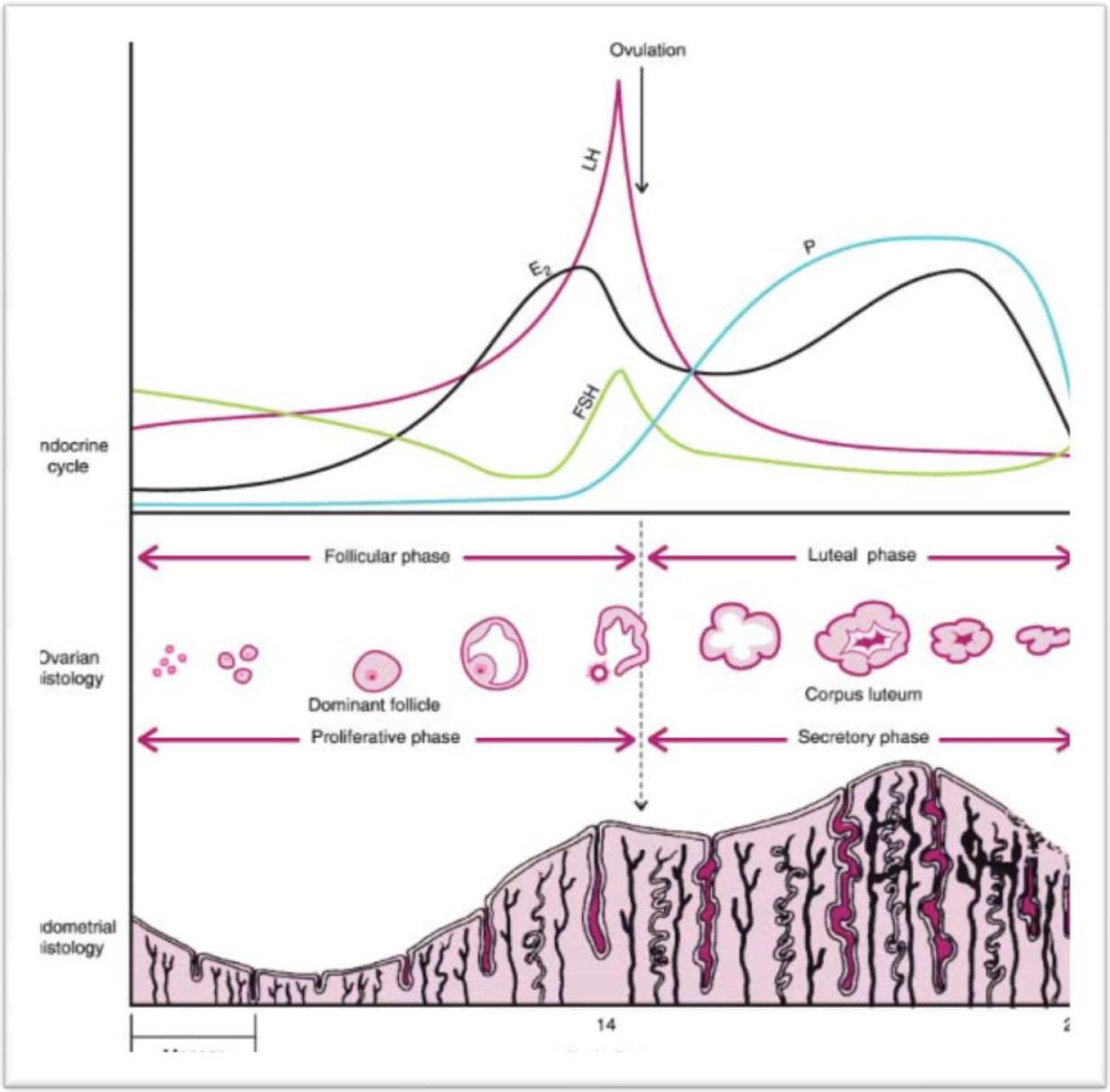
Production of ova

The ovary of the female child at birth contains all the primary oocytes which are scattered amongst the mesenchymal stromal cells of the medulla and cortex. A female fetus contains 7 million oocytes at birth. It is further reduced during childhood so that at puberty the figure is about 400000 oocytes at the onset of menstrual cycle .Of these not more than 500 are designated to mature during the individual's lifetime and the remainder will be lost by degenerative process. During childhood, the ovary grows in size by an increase in stroma .Nevertheless, ova do attempt to ripen from time to time but they fail to complete the process and become blighted [16]

Menstrual Cycle Physiology

This includes the proliferation of the uterine cells and the follicular maturity attained parallels with the production of the hormones. The cycle starts from the development of the follicles to the implantation of the embryo in the uterus or if no fertilization occurs it may lead to mensturation. Various

The Menstrual Cycle.



Pic: 1:- shows the *top panel* shows the cyclic changes of FSH, LH, estradiol (E2), and progesterone (P) relative to the time of ovulation. The *bottom panel* correlates the ovarian cycle in the follicular and luteal phases and the endometrial cycle in the proliferative and secretory phases.

(Jonathan S.Berek.Editor.Novak's Gynecology.Lippincott Williams &Wilkins;2002.13th editionpathological and physiological changes may lead to

disorders in the menstrual cycle, including recurrent miscarriage, infertility and malignancy.

Normal Menstrual Cycle

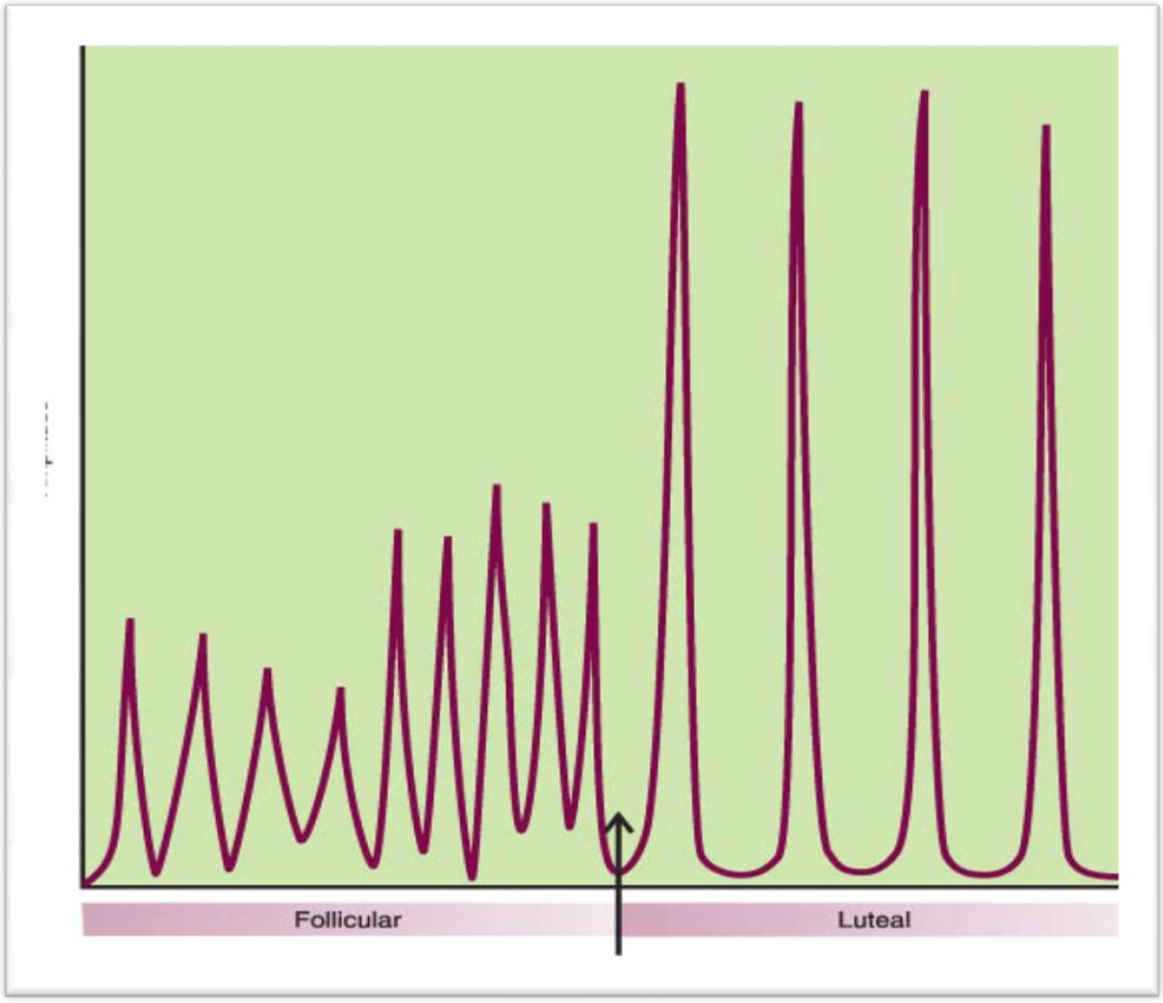
The ovarian and the uterine cycles are the two phase of menstrual cycle. The uterine cycle is divided into the proliferative and secretory phases while the ovarian cycle is divided into follicular and luteal phases. The Follicular phase is the growth and recruitment of a dominant follicle which proceeds to the process of ovulation .The entire process is under the control of hormones. The Normal duration of the menstrual cycle is from 21 to 35 days cycle .This includes the follicular phase with a duration of 10 to 14 days which is in turn has variation depending on the individuals. Luteal phase duration is of 14 days which starts from the day of ovulation to the onset of menses. The menstrual flow is of about two to six days and with an average menstrual blood loss about approximately 20 to 60 ml per cycle. Variations from these would lead to an irregular cycles [17]

Follicular development:- Primodial follicle

Follicular epithelial cells are of single layer and they surround the oocytes measuring about 25-30 μm in diameter. In young individuals there is more of primordial or primary follicles of about 38,000. As these individuals reach the age of 40 their follicular number is reduced to about 8000. At the time of menopause there are only a few follicles or even reduced extremely to one or two in later period of life. A follicle grows upto the antral stage all along the period of fetal life and also during the period of infancy [18]

Growing follicle: At 28 wks of gestation the follicular growth begins and continues upto menopause. Only of about 10% of these follicles are in the active growing period the rest

The pulsatile secretion of GnRH



PIC 2: shows the pulsatile secretion of GnRH in the follicular and luteal phases of the cycle.

(Jonathan S.Berek.Editor.Novak's Gynecology.Lippincott Williams &Wilkins;2002.13th edition)

being inactive. These inactive follicles are called as Primordial follicles or Primary follicles .The active growing follicles is called secondary follicles and the tertiary the preovulatory follicles[16].The secondary follicles are further divided into pre- antral , antral and preovulatory follicles .In the preantral-the follicles grow upto size of 0.4mm and antral follicles grow upto 1-2mm. It require about 50 days to grow to a preovulatory stage . Only one or two follicles attain the preovulatory stage during each menstrual cycle[18.] The pool of primordial follicle is formed in the neonatal period and is considered infinite.

The oocytes continue to grow to antral stage. The selected follicles compete with each other for growth inducing FSH .In response to rise of FSH the preantral follicles begin to secrete estrogens. This have a negative feedback on FSH. Follicles with fewer FSH receptor will not be able to develop further, they will show retardation of their growth and become atretic . Eventually only one follicle will be recruited and is called the dominant follicle. This dominant follicle which will grow up to 20mm in diameter to become the preovulatory follicle which stimulate the estrogen hormone to a high level of for a period of 48 hrs[18.]

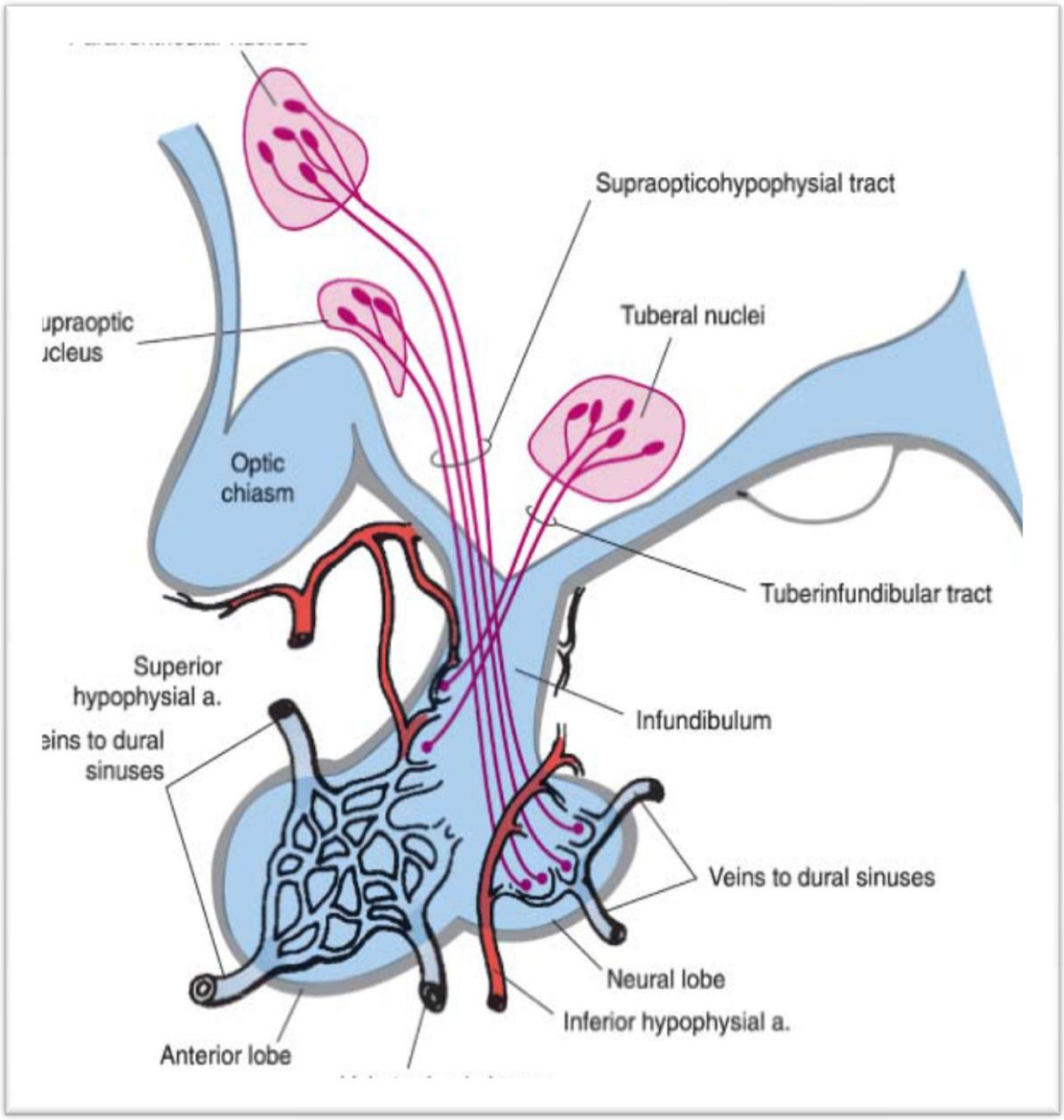
Ovulation

By the end of follicular phase or phase of the 13th day of menstrual cycle.The preovulatory follicle release a single oocyte and it is called ovulation. This needs the midcycle LH surge and it is responsible for the ovulation [20]

Atretic follicles

The primodial follicles which are of about one to two million at the time of birth and only four hundread ovulate among them . The rest of the follicles undergoes a process called atresia and disappears. This follicular atesia is more pronounced in pregnancy, premenarche, and PCOS women.The androstenedione is

Neurologic connections to the pituitary.



PIC 3:- shows the hypothalamus and its neurologic connections to the pituitary.

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produced by the theca interna cells in the absence of granulosa which cannot be converted to estradiol and they are responsible for the androgenic activity in PCOS [18].

Corpus luteaum

Post ovulation the oocytes are discharged from the follicles and the ruptured follicles tends tocollapse .This leads to a scared theca interna called corpus luteum which exists for 14 days after ovulation and begin to degenerate if fertilization does not occur. Then menstrual flow is regulated by estrogens [18] .In the absence of fertilization, post ovulation on the 14 days the corpus luteum disappear.[20]

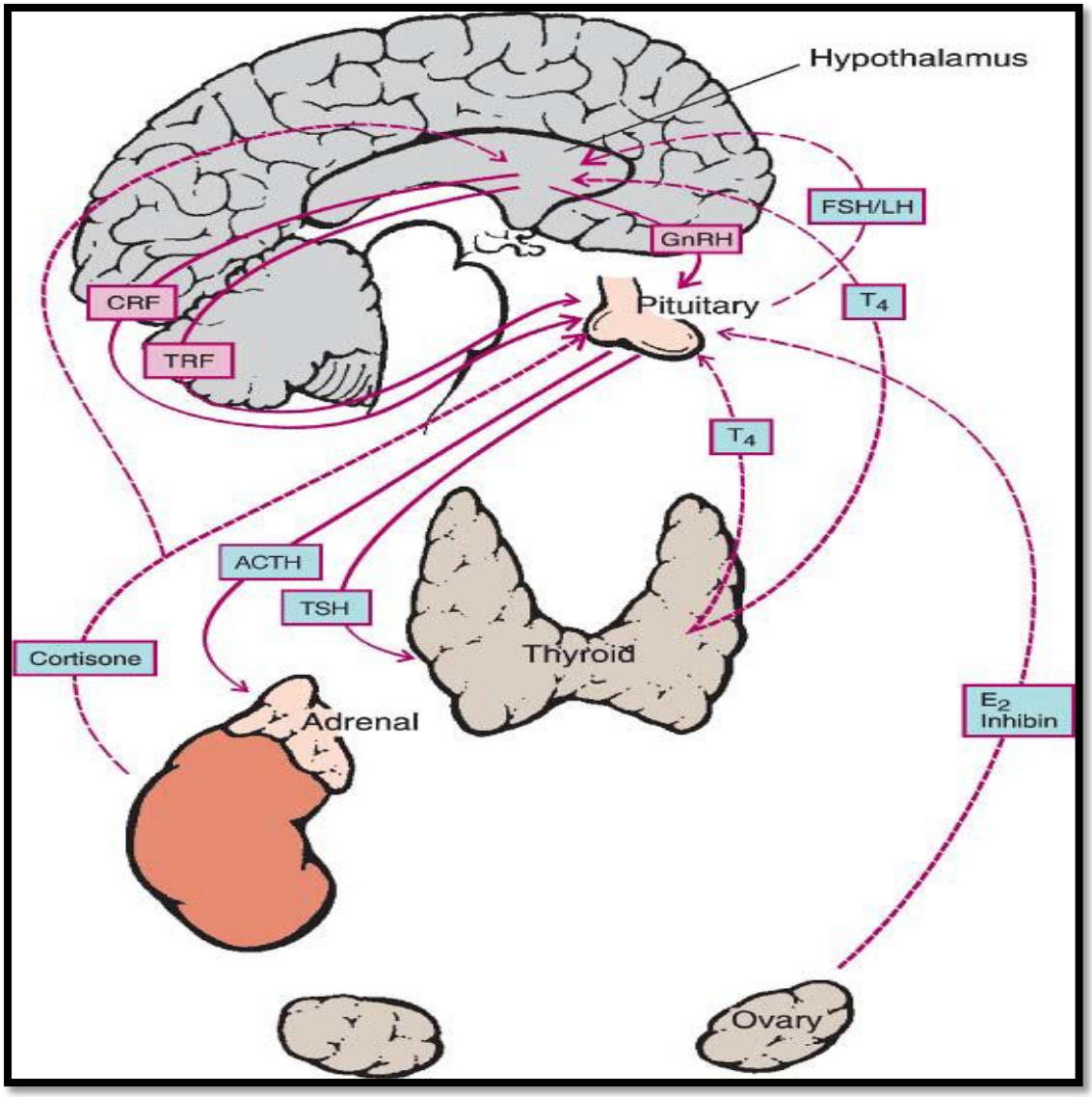
Ovarian hormones:- Estradiol

Estradiol (E2) is a C18 steroid with the presence of phenolic hydroxyl group at C-3 on an aromatic ring, and either a hydroxyl group (estradiol) or a ketone group (estrone) at C-17, which synthesized from its precursor cholesterol.The site of secretion is primarily from the ovary, the placenta, and also in also from adrenal cortex. The secretion is very low after menopause. About 89% of the estrogen is bound to sex hormone binding globulin in blood stream. It is bound to albumin also to a lesser extent. Estrogen activity is affected by the estradiol-receptor complexes which is situated in the target site like the uterus, breast, hypothalamus and pituitary gland. [20]

Pituitary gonadotropins:- Follicle stimulating hormone

Follicle stimulating hormone has two subunits α and β and is a glycoprotein with a molecular mass of 35.5 KDa. The β- subunit is unique while the α-subunit

The hypothalamic secretory function



PIC4 :-showsthe hypothalamic secretory products function as pituitary- releasing Factors that control the endocrine function of the ovaries, the thyroid, and the adrenal gland

(Jonathan S.Berek.Editor.Novak's Gynecology.Lippincott Williams &Wilkins;2002.13th edition)

is similar to other pituitary hormones (20). Follicle Stimulating Hormone (FSH) is under the control of the gonadotropin-releasing hormone (GnRH) which causes its release from the anterior pituitary gland and the hormone is transported via the blood stream to the ovary its target site .FSH stimulates the growth of ovarian follicles in the ovary . FSH also stimulates secretion of estrogen by the follicle cells .[19]

Luteinizing hormon

Luteinizing hormone (LH) is a glycoprotein with a molecular weight of 30 Kilo Daltons [18].The gonadotropin-releasing hormone (GnRH) releases the Lutenizing Hormone from the anterior pituitary and the hormone is taken to the targeted site via the blood. LH then stimulates that follicle until the stage of corpus luteum [19].,Lutenizing Hormone is under the control of the Gonadotrophin Releasing hormone which in turn stimulates the secretion of sex steroids Hormones from the gonads. This operates by a negative feedback loop mechanismon the, sex steroids which inturn inhibits the secretion of GnRH which inturn has a negative effects on gonadotrophs [19].

Prolactin hormone:

Prolactin hormone (PRL) is made of a single polypeptide chain which containing about two hundred amino acid residues and three disulfide bridges and has a considerable structural similarity to human growth hormone. The half-life of PRL, like that of growth hormone, is about 20 minutes[17].PRL is secreted from the anterior

pituitary and its target site of action is on the mammary glands. The main action of the prolactin hormone is the growth and induction of milk secretion during pregnancy and reaching a peak at paturation, PRL secretion is increased by stimulation of the nipples [19].

Thyroid stimulating hormone:

Thyroid stimulating hormone (TSH) is a glycoprotein which is of about 28 to 30 KDa and is secreted from the anterior pituitary. It target site of action is on the thyroid follicular cells present in the thyroid gland [19]. TRH in turn stimulates the anterior pituitary and leads to the secretion of the TSH, which inturn stimulates the release of T4 and T3.Secretion of thyroid-releasing hormone, and in turn the TSH, is inhibited by classical negative feedback loop mechanism. Free or unbound T_3 and T_4 also play a important role by acting via this negative feedback mechanism by maintain the circulating thyroid hormone levels [19].

Poly Cystic Ovarian Syndrome

PCOS is a collection of spectrum of disorder with heterogeneous collection of signs and symptoms which causes changes in the metabolic functions and Reproductive Endocrine in women. PCOS is characterized by irregular menstrual cycles or period of amenorrhoea with or without excess hair growth on face and body (Hirsutism), increase in weight, acene, thinning of the hair on the scalp and associated with ovarian cysts.

The other factors associated with PCOS are obesity, type 2 diabetes, cardiovascular disease, and obstructive sleep apnoea, impaired fertility which increases the risk of diabetes, cardiovascular risk and endometrial cancer which

are the short and long term complications of PCOS in women. In PCOS women the identity, mental health, and health related quality of life are becoming stigmatizing conditions [22].

Prevalence :

The prevalence of Poly Cystic Ovarian Syndrome was estimated to be about 6.6% in adult women aged 18–45 years. However, the prevalence of PCOS was found to be higher in other countries than U.S based on the diagnostic criteria.[15] Estimated PCOS according to the phenotypes, morbidities and prevalence in associated with PCOS due to the lack of large-scale and epidemiological studies on PCOS [23].

The prevalence of other androgens excess disorder using the new diagnostic criteria suggested for diagnosis of PCOS and the most common classic feature of PCOS was androgen excess disorder which was about 30% of patients with clinical hyperandrogenism[24].

There is a necessity for estimation of proportion of women who are affected by PCOS in the population. Earlier studies support the percentage of 2.2-2.6%.Using different available data of three different countries the prevalence is 4.0-11.9%.but in India there is paucity[25].

Clinical features of PCOS:

The common clinical features of PCOS are Oligomenorrhoa and amenorrhoea are signs of anovulation and hirsutism is a sign of hyperandrogenism. Menstural cycles longer than 35 days or less than eight period of menstruation per year is applied for oligo/amenorrhoea. Hirsutism is a sign of androgens excess being the underlying endocrine abnormality. In PCOS the presence of hirsute must be evaluated by clinical

examination and other etiological abnormality must be excluded. Although hirsutism is a frequent feature in PCOS the identification of the etiology needs a systemic approach for evaluation and earlier management [26].The Ferriaman-Gallwey scoring was designed to assess the severity of hirsutism .

The masculine pattern of body hair growth is described in four degrees on eleven different body parts; upper lip , chin ,lower back, chest upper back, lower abdomen, upper abdomen , forearms, arms, thighs and the lower part of the legs. A score greater than 8 was considered hirsute[6].Ovary and adrenal glands produce the androgens. Androgen excess which is the hallmark feature of PCOS and contributor to the, stromal hyperplasia, antral follicle formation and hypervascularity and this androgen excess which is necessary to measure the negative impact on fertility and sub fertility its regulatory benefits in replacement with androgen therapy [27].

Pathophysiology of PCOS:

The pathophysiology for PCOS is multifactorial and polygenic .The phenotype of women with polycystic ovaries (PCO) and the polycystic ovarian syndrome is variable .In understanding the pathophysiology the nature of ovarian dysfunction has to be considered [24].

There is uncertainty in considering the association of inappropriate gonadotrophin secretion that is LH and FSH in PCOS .Hence , twenty four hours pulsatile parameter for serum LH and pituitary gonadotrophin response to I.V bolus GnRH was given and the results showed that BMI does not influence

gonadotrophin in normal cycling women. Taking LH levels and LH/FSH ratio along with BMI is considered beneficial[28].

It is indicated with higher androgen levels, increased prevalence of insulin resistance and more often CVD in first-degree relatives .The correlation of LH / FSH ratio, BMI and clinical manifestations of PCOS was conducted at institution of Infertility treatment and Embryo research. Iraq. The conclusion was negative and no significant correlation was found and the belief that obesity plays a role in pathophysiology of PCOS which was a factor years back[29].

The ovarian morphology is distinct and pathogomonic for its major marker the hyperandrogenemia which is from the theca cells. The entire process from the follicular development to the period of primodial to pre-ovulatory is dependent on the gonadotropins. Polycystic ovaries of the anovulatory cycle had increased number of pre- antral follicle. This is thought to reduce the rate of recruitment from resting follicles. LH is considered to be the cause of hyperandrogenism of PCOS [24]

Along with steroidogenic enzymes in the synthesis of ovarian androgens .There is a possible explanation of the working mechanism of ovarian cautery by laparoscopy as a treatment in PCOS .They established that in PCOS there was thickened ovarian capsule along with dense hyperplastic ovarian stroma and multiple subcapsular cysts of the ovary[30].

In PCOS endocrine disorder associated with chronic anovulation ,an imbalance of LH and FSH results in abnormal estrogen and androgen production

.The serum LH level is high and the serum FSH is low an altered LH/FSH ratio is characteristic[31].The combination of enlarged polycystic ovaries and obesity, oligomenorrhea and hirsutism shows an increased risk for the development of endometrial and possibly breast carcinoma[32].There is large influence of genetic factors to the pathogenesis of PCOS and justified the search for the susceptibility of the gene location [33].

Diagnosis of PCOS

The Revised criteria of 2003 in diagnosing Polycystic Ovarian Syndrome based on the clinical or biochemical evidence of hyperandrogenism, chronic anovulation clinical trial showed that the evidence two out of the three criteria is necessary for the diagnosis[34, 5].

1. Oligo or amenorrhea

2. Biochemical signs or clinical features of hyperandrogenemia

3. transvaginal ultrasonography establishing polycystic ovaries .

There is a wide distribution of heterogenisity of signs and symptoms among women with PCOS and in some subjects PCOS can be expressed over time and can exist without clinical signs and symptoms at present.

PCOS is prevalent in young Reproductive age group where the distribution is of about 20-30%. Polycystic ovaries have several times more primary, secondary and antral follicles compared to non-PCO women. The biological mechanisms behind PCOS was explained less, the condition behind this

seems to be two-factorial. Mainly the intra-ovarian hyperandrogenism which leads to the early follicular growth promotion and leads to excess of follicle with of size 2-5 mm follicle. Studies have also revealed a positive correlation between follicle number and serum Testosterone/Androstendione concentration in PCOS women.

A low aromatase activity due to an insufficient FSH stimulation affected by the synthesis of Estrogens which interferes the selection and growth of a dominant follicle Seconds the hyperandrogenism,. Insulin resistance, secondary to both genetic and lifestyle factors, is associated with anovulation, but probably not its primary cause.

The ESHRE/ASRM sponsored for PCOS consensus workshop which was aimed for the diagnosis and the management of infertility publication in 2004, 2008 respectively. They had discussions on hirsutism and acne include adolescence, the menstrual irregularities, the pregnancy complication, then on the contraception and quality of life, latter with long-term metabolic and cardiovascular health and cancer risks associated with PCOS women.

The criteria needed for the diagnosis of PCOS which differ from adolescents to the reproductive age group women and the risk group with obesity, hirsute and irregular menses which should be classified and treated accordingly in a word of caution with over diagnosis in PCOS. The individual PCOS manifestation in adolescent should be taken in account and treated accordingly [35].

The ovarian volume as the diagnostic criteria for PCOS included 154 women with PCOS under NIH criteria as cases and 57 normal ovarian women as

control. They concluded that ovarian volume 10 cm3 is a good diagnostic criteria for PCOS but even a threshold of 7cm3 with greater than 12 follicles appear to be a best diagnostic criteria and ovarian volume can be used as a surrogate for ovarian volume difficult situations[36].

Ovarian dysfunction:

Women of about one-quarter and one-third of all women with anovulation or menstrual dysfunction have PCOS. The number of antral follicles increases in the ovaries lead to the risk of anovulation. In contrast, the number of ovulatory cycles increases if the number of antral follicles decreases, no matter if it is due to increased age (before menopause) or after ovarian parenchyma-reducing surgery . Women with PCOS usually have increased levels of luteinizing hormone (LH) relative to follicle stimulating hormone (FSH), i.e. the circulating LH to FSH ratio is elevated. The normal LH to FSH ratio which is 1:2 is altered to 2:1 or 3:1. This increase in the LH levels stimulates the theca cells to express the enzyme essential for the production of androgens to a higher extent, thus contributing to androgen excess.

Also insulin stimulates theca cells to secret androgens. The accumulation of the antral follicle along with failure for the selection of the dominant follicle is the characteristic of PCOS .Oligoamenorrhea influence ovarian follicle in PCOS in two different ways .The sensitivity of the follicles to the FSH is excess and leads to more follicular development of small sized follicle 2-5mm diameter. The selection of the dominat follicle form the follicular poll is lost which is under the influence of LH. This explains the follicular arrest in PCOS ovaries.

The excessive stimulation of follicular cells by hormones insulin, LH, or both which contributes for the hyperandrogenism leading to follicular arrest, environment. However, exact mechanisms of follicular arrest are not fully understood.In PCOS women with oligo ovulation exhibit ovarian dysfunction in milder phenotype pattern compared to anovulatory and are favourable in response to the treatment with ovulation induction with clomiphine citrate. But in with recombinant FSH treatment the oligo ovulatory respond less than the anovulatory PCOS [37].

Ultrasound description of polycystic ovary

Transabdominal ultrasound is greatly replaced by transvaginal ultrasound. It was demonstrated that polycystic ovaries were defined as the presence of ≥10 cysts (2 - 8mm diameter), ovarian volume ≥12cm^{3}and bright ecogenic stroma(23 book).Increased stroma echogenicity assessed transvaginally appeared to be exclusively associated with PCOS although this was a subjective appearance rather than a quantifiable measurement. Women with amenorrhoea had similar ultrasound features to those with oligomenorrhea.

The appearance of polycystic ovaries is less important and there is a need to measure the follicular size in PCOS. Polycystic ovaries in premenopausal women are larger than the postmenopausal women.The multiple cysts in the ovary are, with a diameter of 4-10 mm and with normal echogenenicity of the stroma [38]. Although during puberty this is the characteristic appearance of the ovary and also in women recovering from the hypothalamic amenorrhoea- in the above situations it is the follicular growth which continues without recruitment of a dominant

follicle. There is a need to differentiate between multicystic ovaries from polycystic ovaries for the diagnosis and proper management.

In all young women immediately after menarche, irregular menses are common in these years. PCO is a common feature in women with irregular cycles when ultrasonography is done. There is no consensus of PCOS diagnostic criteria during adolescence, emphasizing a polycystic ovary from a multicystic or multifollicular. In adolescents, the ultrasonographic examination is often performed transabdominally, rather than transvaginally, even though the

resolution is better transvaginally. This problem is further magnified by the inferior ultrasound resolution in obesity, and by the changes of ovarian findings with age .

There was significant intra- observers and inter observer variability also using these criteria among inexperienced ultrasonographers, radiologists and gynaecologists, hence there is a need for careful consideration of clinical picture and a supportive biomarker in diagnosing PCOS[24].

The three dimensional ultrasound scanning and pulsed Doppler ultrasonography as a research tool in infertile women. In polycystic ovaries the measurement of ovarian stromal blood flow is a additional and useful parameter. The three- dimensional ultrasound technology can facilitate as a routine in clinical practice [39]. In women undergoing IVF the prediction of ovarian reserve and antral follicular count is of paramount importance in predicting the outcome of pregnancy. The overall performance was assessed by a summary with ROC curve. Conclusion was that ovarian response was similar in the IVF patients with the use

of one single test cannot be used in the assessment of ovarian volume [40].The stage of the disease and the management is largely based on ultrasound findings. Evaluation of ultrasound features of PCOS is dependent on the fact that experience of the specialist and necessary to standardize the description probability[41].

Reproductive consequence of PCOS

The most common cause PCOS is anovulatory cycles of infertility. In majority of women with PCOS anovulation, oligomenorrhoea or amenorrhoea is the main cause and it is due to the irregularities in the menstrual usually. This may be associated with hyperandrogenism either clinical and /or biochemical [24].

Metabolic consequence of PCOS

The condition "the diabetes of bearded women"is a disorder associated with metabolism of carbohydrate and hyperandrogenism . In women with hyperandrogenism and diabetes mellitus skin lesion like acanthosis Nigerians frequently occurred .Additional features include the lipoatrophic diabetes syndromes, pineal hypertrophy, dental precocity, nails are thickened, and associated ovarian enlargement[42].The metabolic evidence is there to justify the inclusion of both non- NIH group PCOS as Poly cystic ovarian subgroup in predicting the risk factor of CVD and T2DM [43].There is a strong genetic relationship to the etiology of PCOS. A considerable progress is made in identifying the susceptibility of gene and mapping studies with TGF-β and insulin signalling, T2DM and obesity susceptibility [44, 45].A retrospective cohort study to show the effect of BMI on the outcome of patients with PCOS. Concluded that

PCOS is a broad syndrome with obese and lean PCOS as two distinct population and have a difference in the IVF outcome and there is a need to differentiate them for the purpose of management and treatment in infertility [46].

Anti Mullerian Hormone (AMH)

Mullerian-inhibiting substance (MIS) was the initial name given for Anti-Mullerian hormone (AMH) [47.It] is a glycoprotein and its molecular weight of AMH is of 140 kD. This belongs to a family of Transforming growth factor β (TGF-β) .It is expressed in the gonads and has a main role in sex differentiation [48,60]. During sex differentiation in the male foetus the AMH is expressed in the Sertoli cells. The testosterone is produced by the induction of the Müllerian ducts and the Leydig cells. The wolffian duct is diffentated into epididymis, Vas deference and the seminal vesicle. The absence of this hormone is responsible for the development of the female reproductive tract, which in turn leads to the differentiation of the mullerian duct into the oviduct, the uterus andthe upper part of the vagina in female foetus [48]. Only from the 36 weeks of gestational age the AMH is, expressed in the ovary from long after the Müllerian ducts female foetus [49,50]. The pre-antral and antral follicles produce AMH from the granulosa cells and involve in the follicular growth and development. Anti Mullerian Hormone in female serum is low at birth but later increase by puberty until adulthood and finally ceases at the period of menopause [50, 51, 52].

The expression of AMH in the follicular fluid is highest in pre-antral and antral follicles sized 2-9 mm, followed by the stage of follicle growth through the next stages of follicle development and is lost in the FSH-dependent stages as well

as in atretic follicles. The primodial follicle pool expresses AMH not from the dominant follicle or atresia cells.

In granulosa cells the up regulation of AMH at the mRNA levels depending on the stages of development of the oocytes [53]. The signalling pathway for AMH has been identified in the gonads and the gonadal cell lines. The main role of AMH on the follicle is inhibition of initial follicle recruitment and reduction of FSH sensitivity on the growing follicles[54, 55]. Remarkably few reports refers that AMH is a predictor of fecundity in women with normal fertility [56].

AMH levels can predict the poor response in ART, and is unrelated to pregnancy outcome [48, 57]. Serum AMH seems to represent a good quantitative measure of the ovarian pool of the primordial follicles, however, whether it represents a quality measure is less founded. Obviously, many aspects of AMH are still unknown and needs to be further explored.

Several studies have recognized the connection between antral follicle count and AMH [58, 59].When the follicle-count diminishes less granulosa cells are available for production of AMH, which does not imply a direct relationship. As age advances there is a decrease in antral follicles and also decrease in both the levels of inhibin and sex steroids the reduced negative feedback acts in and leads to a rise in levels of serum Follicular Stimulating Hormone. Studies looking into the relationship between FSH and AMH under hormonal stimulation in women undergoing IVF. AMH shows a is in negative relationship between serum levels AMH and FSH .However, extension of the FSH-window that occurs during such

gonadotropin-stimulation implies real supra-physiological levels of FSH over a prolonged time.

This in turn, causes an increased recruitment of antral follicles with FSH-induced accelerated growth that at a certain size looses their AMH expression. Hence, the AMH level must drop until a new cohort of follicles in line reach a stage where the AMH production again increases. For a limited amount of time, the amount of granulosa cells able to produce AMH is reduced[60].

Apart from the age-related decline in the AMH production, the ovarian production of this hormone is considered to be relatively stable during pregnancy. A few pre-reviewed papers concerning AMH levels in pregnancy have been published, including three cross sectional studies where one reported stable AMH levels without significant changes throughout the entire pregnancy and two revealed falling levels .A prospective longitudinal study reported a significant decline in AMH levels with advancing gestational length.

This measurement would be useful to calculate the mean value, so the physician and their patients can consider the reproduction option [61].AMH is used to assess the ovarian reserve. So it can assess the relation declare of reproduction capacity [62].

Based on the endocrinal of fetal sex differentiation the defect in androgen and AMH should be suspected if a patient is with ambiguous genitalia [63] AMH is confined to granuosa cells (GC) of primary follicles and is a marker fordiagnosis of ovarian tumour of GC origin.AMH level is found to be increased in 76-93% of

the women with GC tumours. This extends upto the utility of detecting recurrence when elevation of AMH may occurs upto the monitoring period of 16 months [64].

Traditionally, markers for hormonal dosage include demographic parameters, such as age, BMI, serum FSH and Inhibin B and ultrasound markers like antral follicle count and ovarian volume. A common problem connected to these markers has been a low sensitivity and specificity. During the last years papers have focused on AMH as an easy available marker for hormonal dosage in ART in infertility[65]. AMH has a specific role in the ovary and an indicator in women during and after the treatment cycles for cancer therapy [66]. Evaluation of women in treatment and in successful outcome of assisted reproductive technology (ART) before planning treatment it in need to assess the ovarian reserve for good results and to decrease the cycle cancellation rate and the risk of ovarian hyper stimulation syndrome (OHSS) [67].

There is still limited information regarding the use of AMH in a clinical setup. However a marker which reflects the severity of PCOS is them in need In the adolescent period where it is rather difficult to diagnose PCOS by abdominal ultrasound. A marker which can aid in diagnosing the disease in need. This study will aid us to include the measurement of AMH in diagnosing PCOS subjects its role in the choice of the treatment and in further management

Materials and Methods

The present study is a case-control prospective study. The age study population consisted of women aged between 18-40 years. The cases were newly diagnosed PCOS patients. The controls were healthy women having at least one healthy child. Those who attended the Obstetrics and Gynaecology OPD at Chennai medical college hospital and research centre. Irungalur. Trichy.

Period of the study

Women 18 to 40 years old without history of other diseases at their visit between November 2013 to November 2014. Control women were selected from general population with healthy history and have at least one baby. Case and control women were matched for age.

The inclusion criteria

1. Menstrual cycle - regular (length of the cycle 25–35 days, 3-8 days duration of Menstruation)

2. Medications on hormones should be avoided for 3 months**.**

3. Should not be subjected to any surgical procedure in the reproductive system.

4. Patients diagnosed as PCOS (By Rotterdam Criteria)

Exclusion criteria

1. Post Menopausal

2. Thyroid dysfunction

3. Cushing syndrome

4. Congenital adrenal hyperplasia

5. Ovarian tumour

6. Autoimmune disease

Ethical considerations

The researcher obtained the necessary approval to conduct the study from The Chennai Medical College Hospital and Research Centre college ethical committee. Irungalur Trichy. Women were explained about the purpose of this study and an informed written consent was obtained, confidentiality about their results was assured. Their participation was optional.

Blood sample collection and storage:

About 5 ml blood sample were drawn from the median cubital vein from each woman on day three of the cycle or progesterone induced cycle into a plastic pyrogen -free disposable syringe. Transferred into a plastic tube and left for a 20 minutes to allow it to clot. After centrifugation at 3500 rpm for 10 minutes clear serum is obtained or stored at a -20°C until use.

AMH, E2, FSH, LH, Ft3,Ft4 ,TSH, PRL and Total testosterone analyses were carried out at the our central clinical Laboratory.

A. BMI

The body mass index (BMI) was determined by weight and height calculations using the following equation:

BMI = Weight in Kg / Square of height in meters.

According to Indian guidelines, a BMI from 23 to 24.9 is overweight, if the BMI is to be greater ≥ 25 is moderate obesity, and a BMI ≥ 30 is severe obesity.

B. Hirsutism

Ferriman - Gallway score was used to assess the score for hirsutism , any score value >8 was considered to be positive and the presence of hirsutism is confirmed.

C. Ultrasonography

Ultrasound analysis was performed using a trasvaginal US (TVUS) probe on each patient with a 6.5 MHz probe. In unmarried patients a trans abdominal ultrasound was performed.The ultrasound measurement was then obtained by a real-time B scan and was done by a single physician according to the standardized protocol.

A simple formula for prolate ellipse is 0.5XlengthXwidthXthickness was used to calculate ovarian volume. The average of the OV of both ovaries is defined as Ovarian volume per ovary.Antral follicular count was made by a 3 dimensional view and looking for follicle size and number in each dimension.

Uterine size

Uterine diameters are measured in the sagittal plane : maximum length from cervix to fundus X maximum anteroposterior diameter to provide the cross- sectional area.

The follicle number

The follicle number, size and total volume of ovary should be considered .Number of follicles per ovary or single slice of ovary in two diameters number ≥10 or ≥12 or≥15 is considered polycystic. Normal ovaries do not have more than 9 follicles in number.

Size of the follicle

Follicle size 2-6, 2-8, >10mm are taken, as good discriminator between normal and polycystic ovaries .The mean diameter of ovarian volume and the number of follicles with a diameter of 2.0-8.0 mm were used for statistical analysis.

D. Hormone assays

1 . Antimullerian hormone assay

AMH level was determined according to Durlinger *et al*. (1999) method using. UltraSensitive AMH/MIS ELISA AL-105-i

A. Principle of the assay

1. AMH/MIS is a three-step sandwich type immunoassay.

2. The antibody-biotin conjugate binds solid phase of the antibody-antigen complex which then binds to the streptavidin-enzyme conjugate.
3. The complex of antibody-antigen-biotin conjugate-SHRP is bound to the well and is then detected by enzyme-substrate reaction.
4. wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as reference filter measures the degree of enzymatic turnover of the substrate
5. The absorbance measured is directly proportional to the concentration of AMH/MIS in the samples and calibrators.

B. Kit components

A 96 micro titration wells, Six reference standard 0.05, 0.10, 0.25, 1.8, 7.5 and 15 ng/ml

Assay buffer , Wash buffer , diluting solution and the stopping solution.

C. Procedure

1. Micro titration strip was marked for use.
2. Appropriate wells are taken and 20 µL of the standards, controls and samples were added.
3. 100 µl of the MIS/AMH assay buffer were added
4. The wells were incubated at 500-700 rpm and shaken at on an orbital

5. Pattern in a micro plate shaker, for 1hour at room temperature (~25°C).
6. Then each well was aspirated and washed 5 times for 30 seconds with the wash solution using an automatic microplate washer. The plate was inverted aeveral times on an absorbent material to make it dry.
7. Using a precision pipette a 100 µl of the antibody-biotin conjugate solution were added into each well.
8. For one hour at room temperature the wells were incubated and shaken at 500-700 rpm on an micro plate shaker using a precision pipette each well was aspirated and washed 5 times for 30 seconds with wash solution using an automatic micro plate washer or manually
9. To dry it the plate was inverted on an absorbent material using a precision pipette 100 µl of the streptavidin-enzyme conjugate-RTU were added into each well.
10. Wells were incubated and shaken at 500-700 rpm on an orbital
11. Micro plate shaker, for 30 minutes at room temperature (~25°C).
12. Each well were aspirated and washed 5 times for 30 seconds with wash solution using an automatic microplate washer or manually using a precision pipette. Then the plate is dried using a precision pipette 100 µl of the TMB chromogen solution were added into each of the well.

13. Then wells were incubated and shaken at 500-700 rpm on an orbital microplate shaker, for 10-15 minutes at room temperature (~25°C)
14. 100 µl of the stopping solution were added into each well using a precision pipette.
15. The absorbance of the solution in each well was read within 30 minutes, at 450 nm (450-620nm).

D. observation and results

The absorbance for each control, standard, or sample was obtained , and then a standard curve was prepared by plotting the absorbance readings for each of the standards along the Y-axis versus MIS/AMH concentrations in ng/mL along the X-axis, using a linear curve-fit. The MIS/AMH concentrations of the samples were determined from thestandard curve by matching their mean absorbance readings with the corresponding MIS/AMH concentrations.

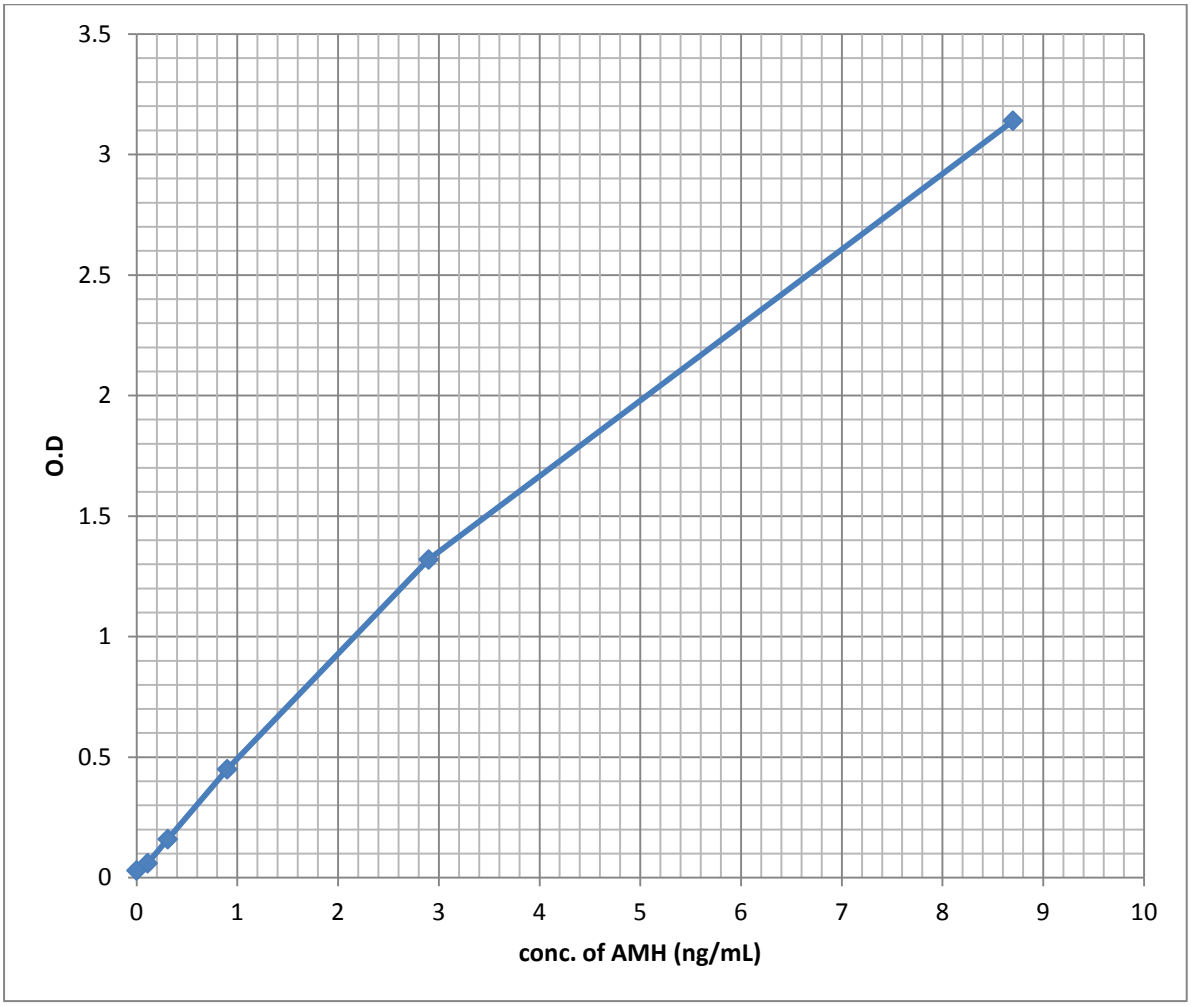
Reference values of AMH in adult females

The range of this hormone on the 3rd day of menstrual cycle is 2.0-6.8 ng/dl .

1. **Testosterone assay:-** Testosterone Test System Product Code: 3725-300

PRINCIPLE: Competitive Enzyme immunoassay (TYPE 7):

Standardization curve for AMH



CONC OF STD (ng/ml)	O.D
0	0.03
0.11	0.06
0.31	0.16
0.9	0.45
2.9	1.32
8.7	3.14

The essential reagent required for a enzyme immunoassay include antibody, enzyme-antigen conjugate native antigen, Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction result between the native antigen and the enzyme-antigen conjugate for a limited number of antibody binding sites, The interaction illustrated by the followed equation.

$$^{Enz}Ag + Ag + Ab_{Btn} \underset{ka}{\overset{ka}{\longleftrightarrow}} Ag\,Ab_{Btn} + {}^{Enz}Ag\,Ab_{Btn}$$

Ab_{Btn} = Biotinylated Antibody

Ag = Native Antigen

Enz Ag = Enzyme-antigen conjugate

Ag Ab Btn = the Antigen-Antibody complex

^{Enz} Ag Ab Btn = Enzyme-antigen conjugate-Antibody complex

K_a = Rate constant of the Association

k-a = Rate constant of the Disassociation

$K=k_a/k_{-a}$ = Equilibrium Constant

On the microwell a simultaneous reaction between the biotin antibody and the streptavidin immobilized occurs. After the aspiration it effects the separation of the antibody bound fraction

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn}$ + Streptavidin cw → immobilized complex

streptavidin cw = streptavidin immobilized on well

Immobilized complex = sandwich complex bound to the solid surface.

The enzyme activity which is in the antibody bound fraction is inversely proportional to the native antigen concentration

REAGENTS

A. Testosterone calibrators- 1ml/vial-icons A-G , serum reference for Testosterone at concentrations of 0 (A) 0.1 (B), 0.5 (C), 1.0 (D),2.5 (E), 5.0 (F) and 12.0 (G) in ng/ml

B. Testosterone Enzyme Reagent -1.0 ml/vial E - Testosterone (Analog) horseradish peroxides (HRP) conjugate ,

C. Steroid conjugate Buffer -7.0 ml

D. Testosterone Biotin Reagent -6.0 ml-anti-Testosterone biotinylated purified rabbit lgG conjuagate in buffer,

E. Steptavidin coated plate -96 wells –One 96-well microplate coated with 1.0 ug/ml steptavidin Wash solution concentrate-20ml

F. Substrate A -7ml/ tetramethyl benzidine (TMB) in buffer.

G. Substrate B – 7ml/ vial –hydrogen peroxide (H2 O2) in buffer .

H. Stop solution – 8ml/vial-a strong acid (1N HCI).

PROCEDURE

1. Control and patient specimen are formatted in microplates wells for each serum reference to be assayed in duplicate.
2. In the assigned well Pipette 0.010ml (10ml) of the appropriate serum reference control or specimen.
3. Testosterone Enzyme Reagent of 0.050 ml (50ul) is added to all the well for 20-30 seconds Swirl the microplate gently
4. To all wells add Testosterone Biotin Reagent of 0.050 ml (50ul) for 20-30 seconds to mix Swirl the microplate .
5. At room temperature cover and then incubate for one hour.
6. Aspirate the content of microplate and discard and with absorbent paper blot the plate dry.
7. Add 350ul of wash buffer and aspirate. Repeat the same for two times for a total up to three wash.
8. To all wells add 0.100 ml (100ml) of working substrate at room temperature incubate for 15 minutes.

9. Stop solution of 0.050ml (50ul) is added of to each well. Mix for 15-20 seconds.
10. Read the absorbance in each well at 450nm

RESULTS.

Absorbance for each standard, control, or sample was obtained, and then a standard curve was prepared by plotting the absorbance values for each of the standards along the Y-axis versus the X-axis the standard concentrations in pg/ml. A best-fit curve connecting the points is done. The mean absorbance value estimated for each sample using the corresponding concentration of testosterone ng/dl from the standard curve

Reference range values of the Testosterone in adult females:

The normal range of Testosterone during the early follicular phase in adult females is between 15.0 – 80.0 ng/dl

3. Estradiol assay

Estradiol (E2) Test Systems Code of the Product : 4925-300

PRINCIPLE

Delayed Competitive Enzyme linked Immunoassay

The biotinylated Antibody with a serum containing the antigen, a reaction results between the antigen and the antibody. The interaction is illustrated by the following equation

$$Ag + Ab_{Btn} \longrightarrow Ag\ Ab_{Btn}$$

Ab = Biotinylated antibody

Ag = Antigen

AgAb = the Immune Complex

The enzyme conjugate is added After a short incubation. Upon the addition of the enzyme conjugate, competition reaction results between the enzyme analog and the antigen in the sample for a limited number of antibody binging sites (not consumed in the first incubation).

$$^{Enz}Ag + Ag + rAb_{Btn} \underset{K_{-a}}{\overset{K_a}{\longleftrightarrow}} Ag\ Ab_{Btn} + {}^{ENZ}AgAb_{Btn}$$

Enz Ag b= Enzyme-antigen Conjugate (Constant Quantity)

Enz Ag Ab = Enzyme-antigen Conjugate-Antibody Complex

Ab Btn = Biotinylated antibody not reacted in first incubation

K a = Rate Constant of Association

K-a = Rate Constant of Disassociation

K = Ka/ ka = Equilibrium Constant

After aspiration.Streptavidin is immobilized on the microwell this effects the separation of the antibody bound fractions.

$AgAb_{Btn} + {}^{ENZ}AgAb_{Btn}$ + Streptavidin $\longrightarrow$ the Immobilized complex

Streptavidin = Streptavidin immobilized on the well;

Immobilized complex = Sandwich complex bound to the solid surface

The enzyme activity in the antibody bound fraction is inversely proportional to the native antigen concentration.

REAGENTS

A. Estradiol Calibrators -1ml .
B. Add the Reference serum for estradiol at concentration of 0 (A), 20 (B), 100 (C), 250 (D), 500 (E), 1500 (F) and 3000 (G) in pg/ml.
C. Estradiol Enzyme Reagent -6.0 ml horseradish peroxides (HRP)
D. Estradiol Bioting Reagent -6.0 ml -anti-estradiol biotinylated purified rabbit lgG
E. 96 Wells Streptavidin Coated Plates Each well is coated with 1.0 ng/ml strep avidin.
F. Wash solution -20ml
G. Substrate Reagent – 12ml -teramethylbenzidine (TMB) and hydrogen peroxide (H2 O2) in buffer
H. Stop Solution -8 ml of strong acid (H2 SO4) store at 2.30 C

PROCEDURE

1. Serum reference control or specimenof about 0.025 ml (25ul) is Pipetted into the well.
2. Estradiol Biotin Reagent of 0.050 ml (50ul) is added.
3. For 20-30 seconds to mix swirl the microplate gently.
4. At room temperature incubate after Covering for half hour.
5. Estradiol Enzyme Reagent of 0.050 ml (50ul) is added in the wells.
6. For 20-30 seconds swirl the microplate gently to mix at room temperature Cover and incubate for 1 hour and 30 mins.
7. Aspirate the content of the microplate and discard
8. With absorbent paper blot the plate dry.
10. Add 350µl of wash buffer -aspirate the content Repeatby three additional times.
11. Add 0.050ml (100ul) of substrate solution to all
12. Incubate at room temperature for twenty (20) minutes
13. Add 0.050ml (50ul) of stop solution to each well

The absorbance is read in each well at 450nm

RESULTS:

Absorbance for the control, standard, or sample was obtained. by plotting the absorbance readings a standard curve was prepared for each of the standards plotting

the standard concentrations in pg/ml along the X-axis and absorbance reading along the Y-axis . Connect the best of the points. From the standard curve the mean absorbance of the value for each of the sample was used to determine the concentration of estradiol in pg/ml

E. Reference value of E2 in adult females

The normal range of these hormones in adult females during early follicular phase is between 25-100 pg/ml

4. Follicles Stimulating Hormone assay

The Follicles Stimulating Hormone (FSH) *Product Code 425-300*

PRINCIPLE

The Immuno enzymometric assay method

The immobilization of the antibody takes place during the assay at the surface of a microplate well by the interaction of the biotinylated monoclonal anti-FSH antibody and the streptavidin coated well. They form a soluble sandwich antigen antibody complex.

The interaction is illustrated by the following equation

$$^{Enz}Ab_{(p)} + Ag + {}^{Btn}Ab_{(m)} \underset{K\text{-}a}{\overset{Ka}{\longleftrightarrow}} {}^{Enz}Ab_{(p)} - Ag_{FSH} - {}^{Btn}Ab_{(m)}$$

Ab = Biotinylated Monoclonal Antibody

Ag = the Native Antigen

Enz Ab = Enzyme labeled Antibody

Enz ABb = gfsh Ab = Antigen –Antibodies sandwich compiled

k-a = Rate Constant of Association

k-a = Rate Constant of Dissociation

streptavidin and biotinylated antibody complex is deposited to the well through the high affinity reaction .This interaction is illustrated below.

Enz Ab gfsh = Ab + Streptavidin =Immobilized complex

Streptavidin = Streptavidin immobilized on well

Immobilized complex = sandwich complex bound to the solid surface.

After aspiration, the antibody-bound fraction is separated from unbound antigen.

The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration.

REAGENTS;- Materials Provided:

1. FSH Calibrators -1 ml

FSH Antigen at levels of 0 (A) 5 (B), 0 (C), 25(D), 50(E) and 100 (F)

2. FSH Enzyme Reagent – 13 ml -enzyme labloed antibody, biotinylated monoclonal mouse lgG

3. Streptavidin Coated Plate -96 wells

4. Wash solution concentrate -20 ml

5. Substrate A – 7.0 ml- tetramethylbenzidine (TMB) in buffer

6. Substrate B -7.0 ml- hydrogen peroxide (H2,O2)

7. Stop solution – 8ml- strong acid (1N HCI)

PROCEDURE

1. Appropriate serum reference control is pipetted of about 0.050 ml (50ul) of into the assigned well.

2. FSH-Enzyme Reagent solution of 0.100ml (100ul) is added to all wells.

3. For 20-30 seconds to mix then swirl in the microplate.

4. At room temperature incubate 1 hour.

5. Aspirate the content and dry the microplate.

6. Add 350ul of wash buffer and repeat the procedure 3 times.

7. Working substrate solution of 0.100 ml (100ul) is added to all wells

8. Incubate at room temperature for fifteen (15) minutes.

9.Add 0.050 ml (50ul) of stop solution to each well and gently mix for 15-20 seconds

10. Read the absorbance in each, well at 450nm

RESULTS

Absorbance for the control, standard, or sample was obtained. by plotting the absorbance readings a standard curve was prepared for each of the standards plotting the standard concentrations in pg/ml along the X-axis and absorbance reading along the Y-axis . Connect the best of the points. From the standard curve the mean absorbance of the value for each of the sample was used to determine the concentration of FSH in IU/ml from the standard curve.

E. Reference values of FSH in adult females

The normal range of Follicle stimulating hormones in adult females during early follicular phase is between 1.4- 9.9 mIU/mL

5. Luteinizing Hormone assay

Luteinizing Hormone (LH) Code of the Product: 625 -300

PRINCIPLE

Immuno enzymometric assay. The biotinylated monoclonal anti-LH antibody is added to the streptavidin in the microplate .Upon mixing monoclonal biotinylated antibody, the enzyme labelled antibody a soluble sandwich complex is formed. The interaction is illustrated by the following equation.

$$^{Enz}Ab_{(p)} + Ag_{LH} + {}^{Btn}Ab_{(m)} \underset{K\text{-}a}{\overset{k\text{-}a}{\longleftrightarrow}} {}^{Enz}Ab_{(p)} - Ag_{LH} - {}^{Btn}Ab_{(m)}$$

Ab = Biotinylated Monoclonal Antibody

Ag = Native Antigen

Enz Ab = Enzyme labeled Antibody

Enz Ab-Ag-Ab = Antigen- Antibodies sandwich Complex

k-a = the rate constant of Association

k-a = the rate Constant of Dissociation

Streptavidin and biotinylated antibody the complex is deposited to the well. This interaction is illustrated below.

Enz Ab – Ag –Ab + Streptavidin = Immobilized complex

Streptavidin = Streptavidin immobilized on well

Immobilized complex = Antibodies –Antigen sandwich bound

Aspiration, the antibody-bound fraction is separated from the unbound antigen. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration.

REAGENTS

LH Calibrators- 1ml -LH Antigen at levels of 0(A), 5 (B), 25(C), 50 (D), 100 (E) and 200 (F)mlU/ml.

A. LH Enzyme Reagent -13 ml - enzyme labelled affinity purified antibody, biotinylated monoclonal mouse lgG.

B. Streptavidin coated plate -96 wells.

C. Wash solution concentrate -20 ml.

D. Substrate A – 7 ml- tetramethyl benzidine (TMB) in buffer.

E. Substrate B -7 ml -hydrogen peroxide (H2 O2) in buffer.

F. Stop solution - 8ml - strong acid (1N HCI).

PROCEDURE

1. serum reference, control and patient specimen in a micro plate wells.
2. Pipette 0.050 ml (50ul) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.00ml (100ul) of LH-Enzyme Reagent.
4. 20-30 seconds Swirl the micro plate.
5. At room temperature incubate for 1 hour.
6. Aspirate the microplate and discard the content dry the microplate.
7. Add 350ul of wash buffer for a total of 3 washes.
8. Working substrate solution of 0.00ml (100ul) is added to the well.
9. At room temperature incubate for fifteen minutes.
10. Stop solution of 0.050ml is added and gently mixed for 15- 20 seconds.
11. Read the absorbance in each well at 450nm

RESULTS.

Absorbance for the control, standard, or sample was obtained. by plotting the absorbance readings a standard curve was prepared for each of the standards plotting the standard concentrations in pg/ml along the X-axis and absorbance reading along the Y-axis . Connect the best of the points. From the standard curve the mean absorbance of the value for each of the sample was used to determine the concentration of LH in IU/ml from the standard curve

E. Reference values of LH in adult females

The normal range of Luteinizing hormone in adult females during early follicular phase is between 1.7 - 15 mIU/ml

6. Prolactin Hormone assay

THE Prolactin Hormone (PRL) Code of the Product : 725-300

PRINCIPLE

Immunoenzymometric assay (TYPE 3):

The streptavidin coated on the well and biotinylated monoclonal anti– PRL antibody is added. A soluble sandwich complex is formed by mixing monoclonal biotinylated antibody, and the enzyme labelled antibody and a serum containing the native antigen reaction results between the native antigen and the antibodies. The interaction is illustrated by the following equation.

$$^{Enz}\ Ab_{(P)} + Ag_{PRL} + {}^{Btn}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\longleftrightarrow}} {}^{Enz}\ Ab_{(P)} \text{ -- } Ag_{PRL} - {}^{Btn}Ab_{(m)}$$

Ab = Biotinylated Monoclonal Antibody

Enz Ab = the Native Antigen

Enz Ab = the Enzyme labelled Antibody

Enz Ab -Ag -Ab = Antigen – Antibodies Sandwich Complex

K – a = the Rate Constant of Association

K – a = the Rate Constant of Dissociation

Simultaneously, the complex of streptavidin and biotinylated antibody is deposited to the well. This interaction is illustrated below.

Enz Ab - Ag -- Ab + Streptavidin = immobilized complex

Streptavidin = Streptavidin immobilized on well

Immobilized complex = Sandwich complex bound to the well.

After equilibrium is attained, the antibody – bound fraction is separated from unbound antigen by decantation of aspiration

REAGENTS

A. PRL Calibrators - 1 ml - PRL antigen in human serum at levels of 0(A), 5(B), 10(C), 25(D), 50(E), and 100 (F) ng/ml

B. PRL Enzyme –Reagent – 13ml - enzyme labelled antibody, biotinylated monoclonal mouse igG

C. Streptavidin Coated Plate – 96 wells

D. Wash solution concentrate -20 ml

E. Substrate A – 7ml - tetramethylbenzidine (TMB) in buffer

F. Substrate B- 7ml - hydrogen peroxide (H2,O2) in buffer.

G. Stop solute on – 8ml -a strong acid (1N HCI).

PROCEDURE

1. Format the microplate wells for each serum reference control and patient specimen.
2. Pipette 0.025 ml (25ul) of the appropriate serum reference control or specimen into the assigned well.
3. PRL Enzyme Reagent solution of 0.100 ml (100ul) added to the wells.
4. For 20-30 seconds swirls the microplate gently.At room temperature incubate 1 hour.
5. Aspirate the microplate and discard and dry the plot by absorbent paper.
6. Add 350ul of wash buffer and repeat the procedure for 3 wash
7. Working substrate solution of 0.100 ml (100ul) is added.
8. At room temperature incubate for fifteen (15) minutes

9. Stop solution of 0.050ml (50ul) is added and mix for 15-20 seconds
10. Read the absorbance in each well at 450nm

E. Reference values of PRL in adult females

The normal range of Prolactin hormones in adult females in early follicular phase is between 0.9- 25 mIU/ml**.**

Results and statistics

Age group	Cases N (%)	Controls N (%)	Total N (%)
<20 years	3 (10)	3 (10)	6 (20)
21- 30 years	18 (60)	18 (60)	36 (60)
31 - 40 years	9 (30)	9 (30)	18 (30)
Total	30 (100)	30 (100)	60 (100)

Mean age: 28.63 years

Standard deviation: 5.64 years

Minimum: 18 years

Maximum: 40 years

Table 1 Age distribution of the study population (n=60)

Age distribution

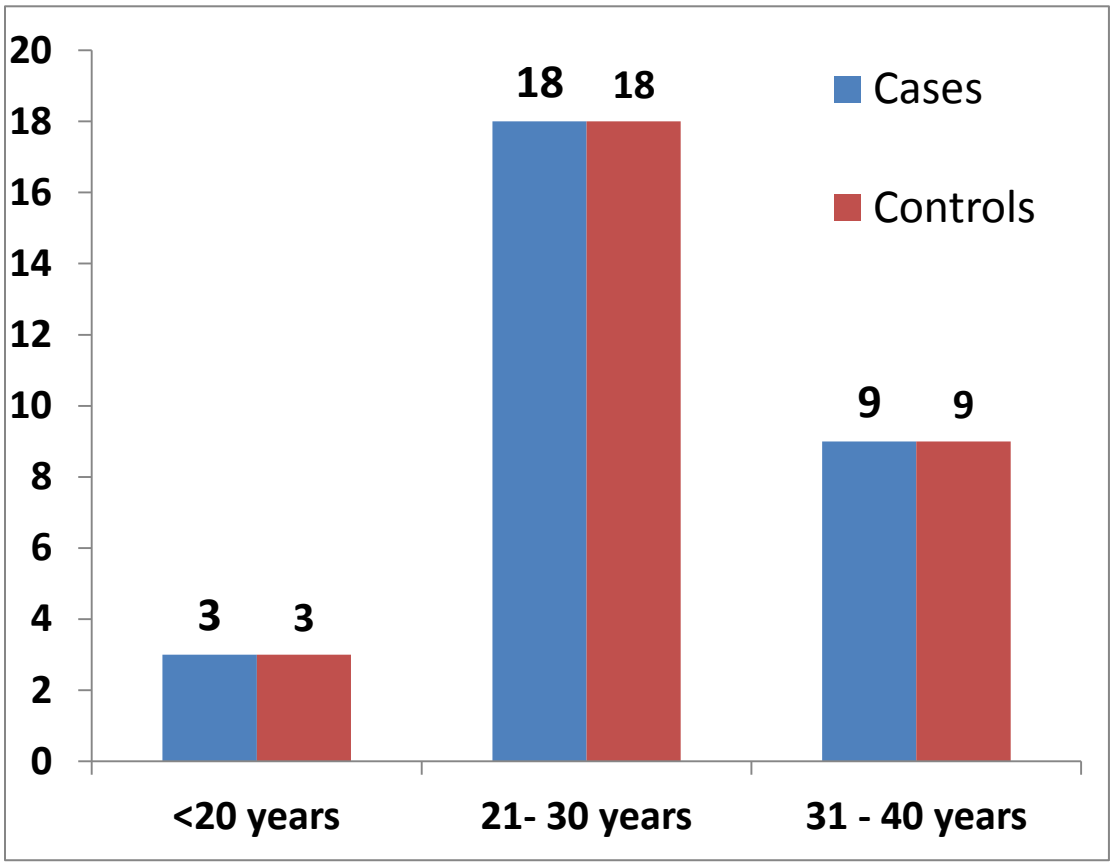


Figure 1: Age distribution of the study population (n=60)

Comparison of age

Student "t" test

Group	Mean Age	Std. Deviation	Mean difference	*p* value	95% confidence interval
Cases (30)	28.50	5.625	-0.267	0.857	-3.210 to 2.677
Controls (30)	28.77	5.764			

Subjects in the Cases group were not so different from subjects in control group with the mean age difference being 0.267 and this mean difference was not statistically significant. Hence both the cases and controls were comparable in terms of age.

Table 2 Comparison of age among cases and controls (n=60)

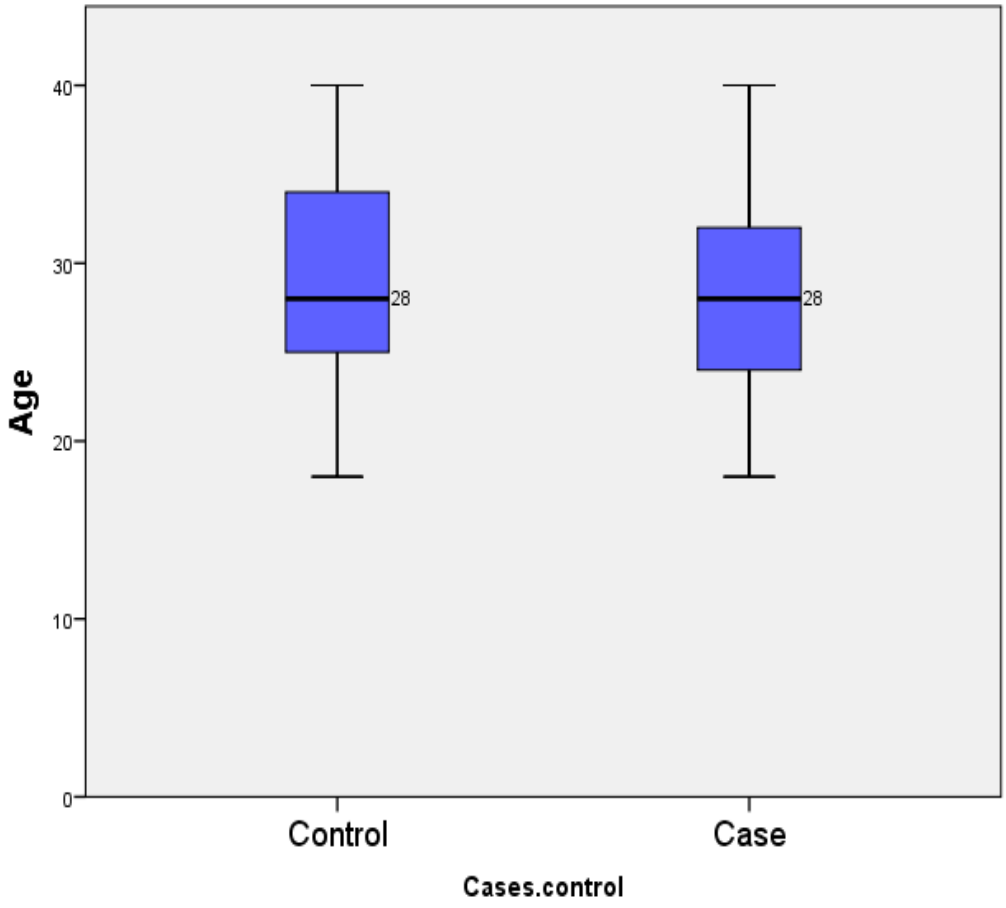


Figure 2: Box plot comparison of age among cases and controls (n=60)

Comparison of LH hormone levels (mIU/ml)

Student "t" test

Group	Mean LH (mIU/ml)	Std. Deviation	Mean difference	*p* value	95% confidence interval
Cases (30)	10.60	3.24	3.21	0.001*	1.299 to 5.120
Controls (30)	7.39	4.10			

**statistically significant at 0.05 level*

Subjects in the Cases group had a mean LH level of around 10mIU/ml while subjects in control group had a mean LH level of around 7mIU/ml and this mean difference was statistically significant.

Table 3: Comparison of LH hormone levels (mIU/ml) among cases and controls (n=60)

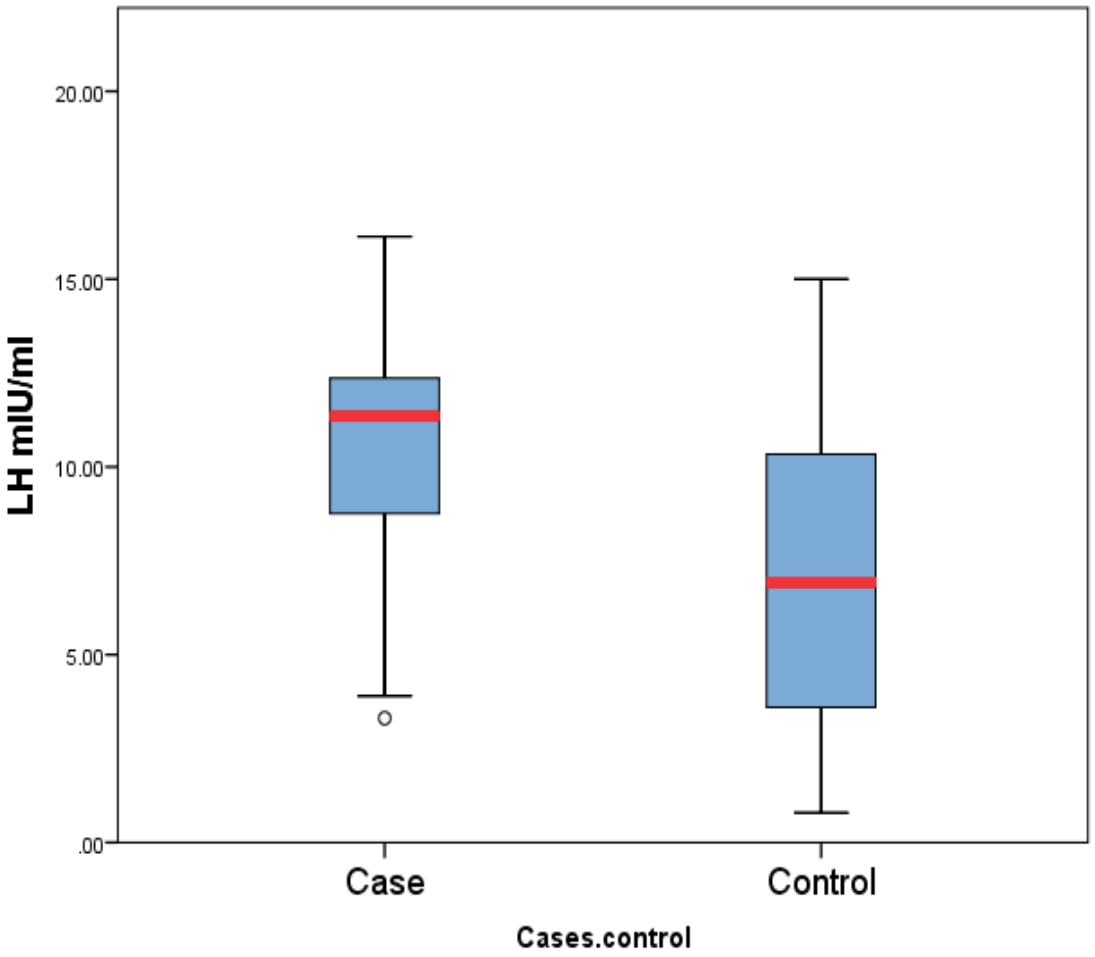


Figure 3: Box plot comparison of LH hormone levels (mIU/ml) among cases and controls (n=60)

Comparison of FSH hormone levels (mIU/ml)

Student "t" test

Group	Mean FSH (mIU/ml)	Std. Deviation	Mean difference	*p* value	95% confidence interval
Cases (30)	5.60	1.99	0.059	0.925	-1.191 to 1.309
Controls (30)	5.54	2.78			

The difference in mean FSH levels between cases and controls was very minimal of about 0.06 mIU/ml and this mean difference was not statistically significant.

Table 4 Comparison of FSH hormone levels (mIU/ml) among cases and controls (n=60)

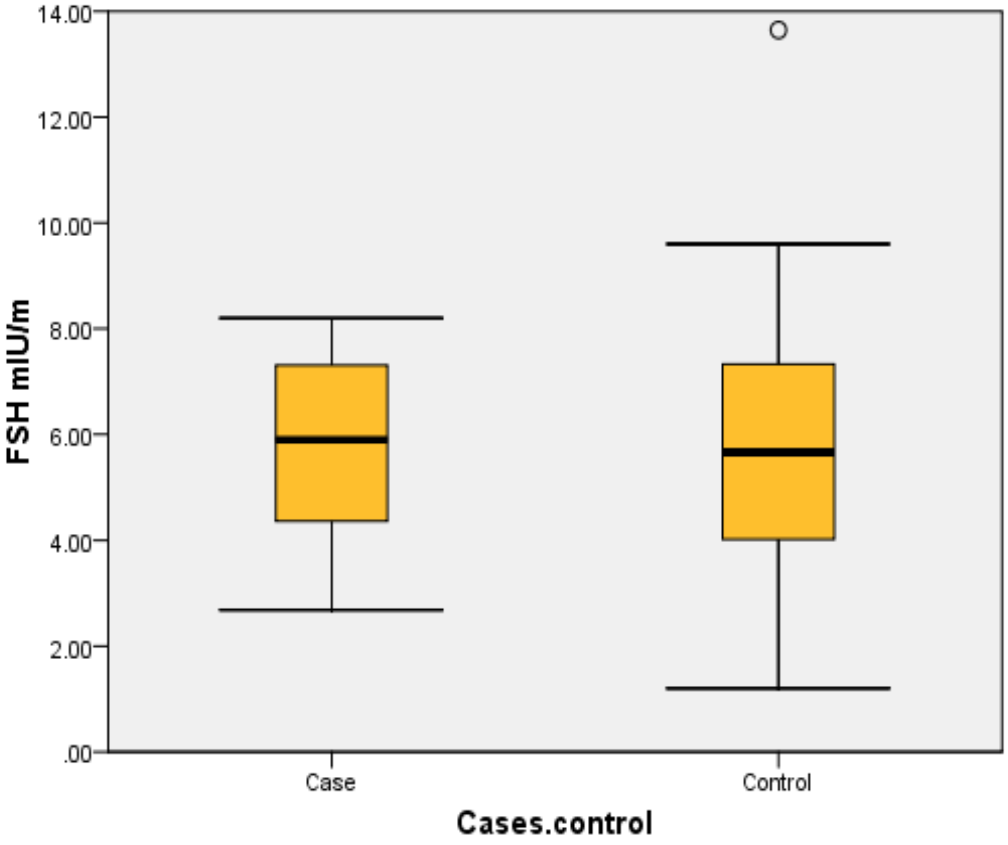


Figure 4: Box plot comparison of FSH hormone levels (mIU/ml) among cases and controls (n=60)

Comparison of Testosterone levels (ng/ml)

Student "t" test

Group	Mean testosterone (ng/ml)	Std. Deviation	Mean difference	*p* value	95% confidence interval
Cases (30)	73.02	19.43	23.07	<0.001*	14.48 to 31.66
Controls (30)	49.94	13.21			

**statistically significant at 0.05 level*

Subjects in the Cases group had a mean testosterone level of around 73ng/ml and subjects in control group had a mean testosterone level of around 50ng/ml and this mean difference was statistically significant.

Table 5 Comparison of Testosterone levels (ng/ml) among cases and controls (n=60)

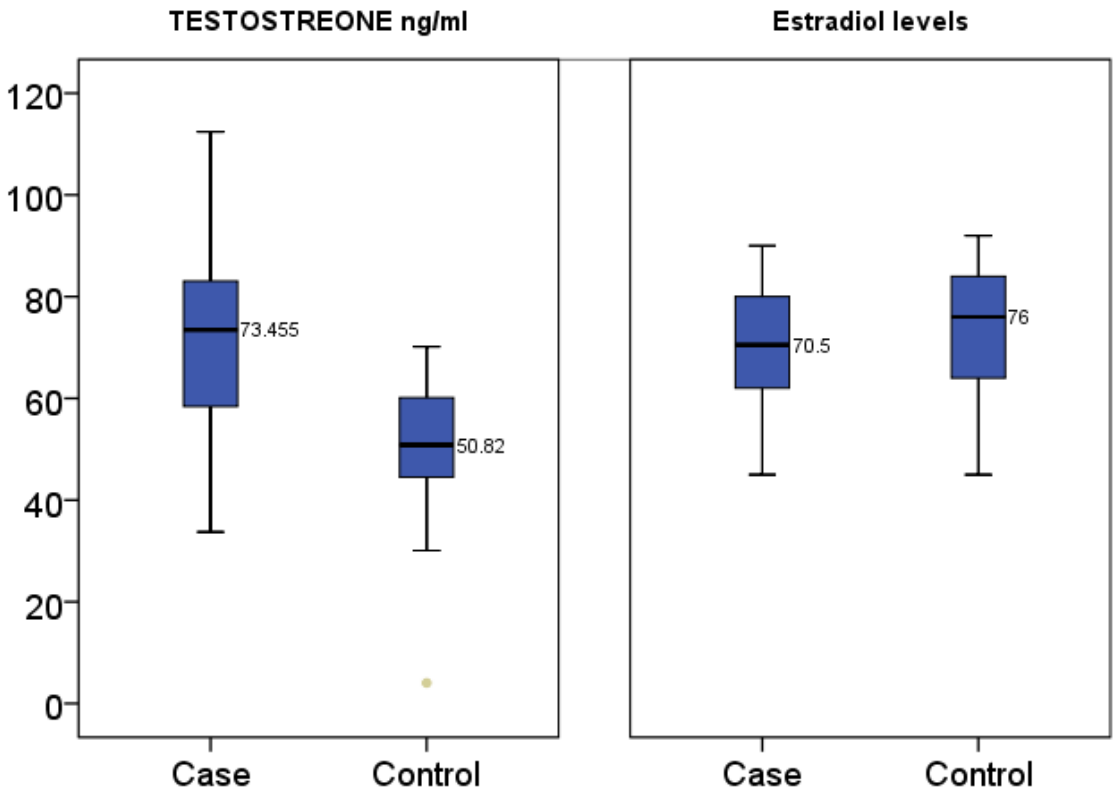


Figure 5: Box plot comparison of testosterone (ng/ml) and Estradiol hormone levels (pg/ml)among cases and controls (n=60)

Comparison of E_2 level (pg/ml)

Student "t" test

Group	E_2 level (pg/ml)	Std. Deviation	Mean difference	p value	95% confidence interval
Cases (30)	71.18	10.88	-2.51	0.409	-8.57 to 3.54
Controls (30)	73.70	12.50			

Subjects with PCOS had a mean estrogen (E_2) level of 71 units and subjects in control group had a mean level of around 73 units and this mean difference was not statistically significant.

Table 6 Comparison of E_2 level (pg/ml) among cases and controls (n=60)

Comparison of Prolactin levels (ng/ml)

Student "t" test

Group	Mean prolactin (ng/ml)	Std. Deviation	Mean difference	*p* value	95% confidence interval
Cases (30)	12.57	5.29	3.39	0.007*	5.82 to 0.95
Controls (30)	9.18	4.04			

**statistically significant at 0.05 level*

Subjects in the Cases group had a mean prolactin hormone level of around 12ng/ml and subjects in control group had a mean level of around 9ng/ml and this mean difference was statistically significant.

Table 7 Comparison of Prolactin levels (ng/ml) among cases and controls (n=60)

Comparison of total antral follicular count

Student "t" test

Group	Combined mean AFC	Std. Deviation	Mean difference	*p* value	95% confidence interval
Cases (30)	30.20	3.36	22.0	<0.001*	20.536 to 23.464
Controls (30)	8.20	1.62			

**statistically significant at 0.05 level*

Subjects in the Cases group had a mean combined antral follicular count of around 30 and subjects in control group had a mean level of around 8 and this mean difference was statistically significant.

Table 8 Comparison of total antral follicular count (combined AFC of both right and left ovaries) among cases and controls (n=60)

Comparison of average ovarian volume (cm^3)

Student "t" test

	Average ovarian Volume (cm^3)	Std. Deviation	Mean difference	*p* value	95% confidence interval
Cases (30)	15.13	2.67	9.99	<0.001*	8.96 to 11.03
Controls(30)	5.14	0.87			

**statistically significant at 0.05 level*

Subjects with PCOS had a average ovarian volume of around $15cm^3$ and subjects in control group had a average ovarian volume of around $5cm^3$ and this mean difference was statistically significant.

Table 9 Comparison of average ovarian volume (cm^3) among cases and controls (n=60)

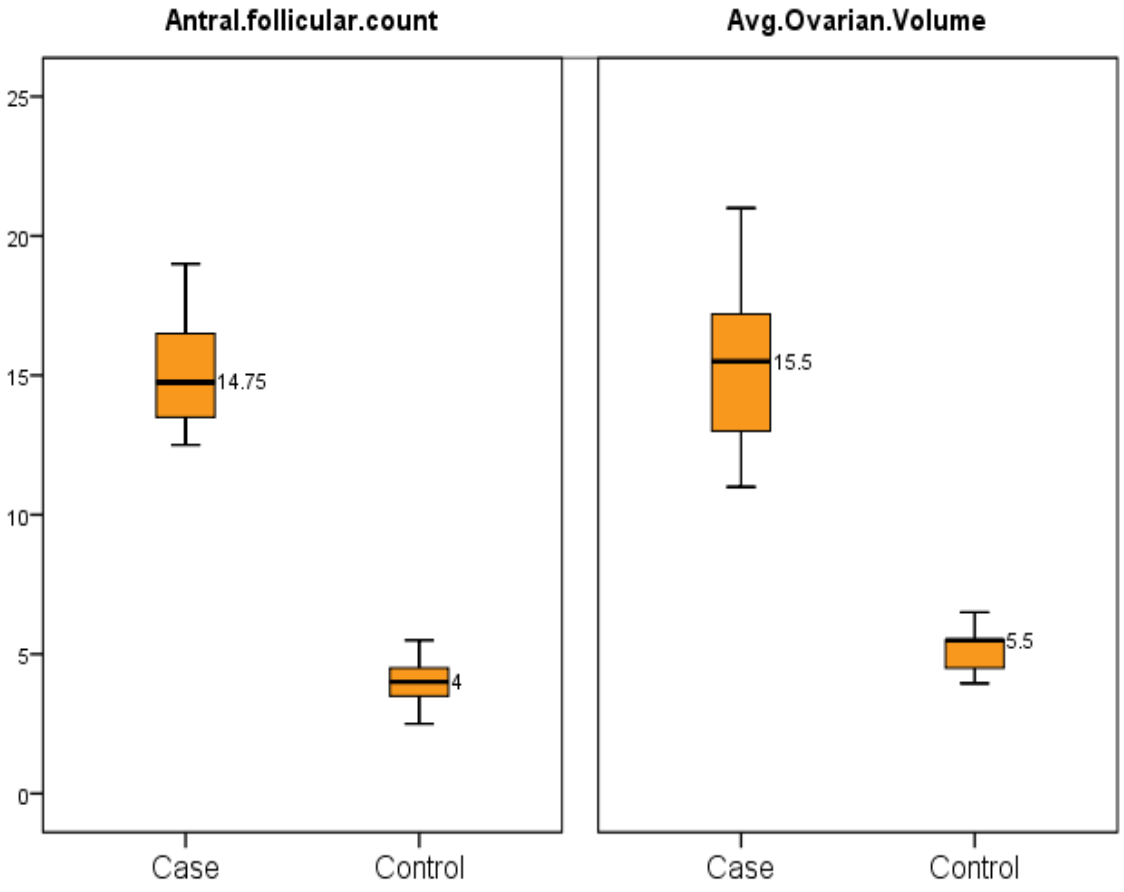


Figure 6: Box plot comparison of antral follicular count and average ovarian volume (cm^3) among cases and controls (n=60)

Comparison of body mass index (BMI) (kg/m^2)

Student "t" test

Group	BMI (kg/m^2)	Std. Deviation	Mean difference	*p* value	95% confidence interval
Cases (30)	28.46	4.78	2.94	0.031*	0.272 to 5.610
Controls (30)	25.52	5.51			

**statistically significant at 0.05 level*

Subjects with PCOS had a BMI of around 28 kg/m^2 and subjects in control group had a mean level of around 25 kg/m^2 and this mean difference was statistically significant.

Table 10 Comparison of body mass index (BMI) (kg/m^2) among cases and controls (n=60)

Comparison of anti-mullerian hormone (AMH) levels (ng/ml)

Student "t" test

Group	Mean AMH (ng/ml)	Std. Deviation	Mean difference (ng/ml)	*p* value	95% confidence interval
Cases (30)	11.28	5.29	7.62	<0.001*	5.58 to 9.66
Controls (30)	3.65	1.46			

**statistically significant at 0.05 level*

Subjects in the Cases group had a mean anti-mullerian hormone level of around 11ng/ml and subjects in control group in case the mean level of AMH was around 3ng/ml and this mean difference was statistically significant.

Table 11 Comparison of anti-mullerian hormone (AMH) levels (ng/ml) among cases and controls (n=60)

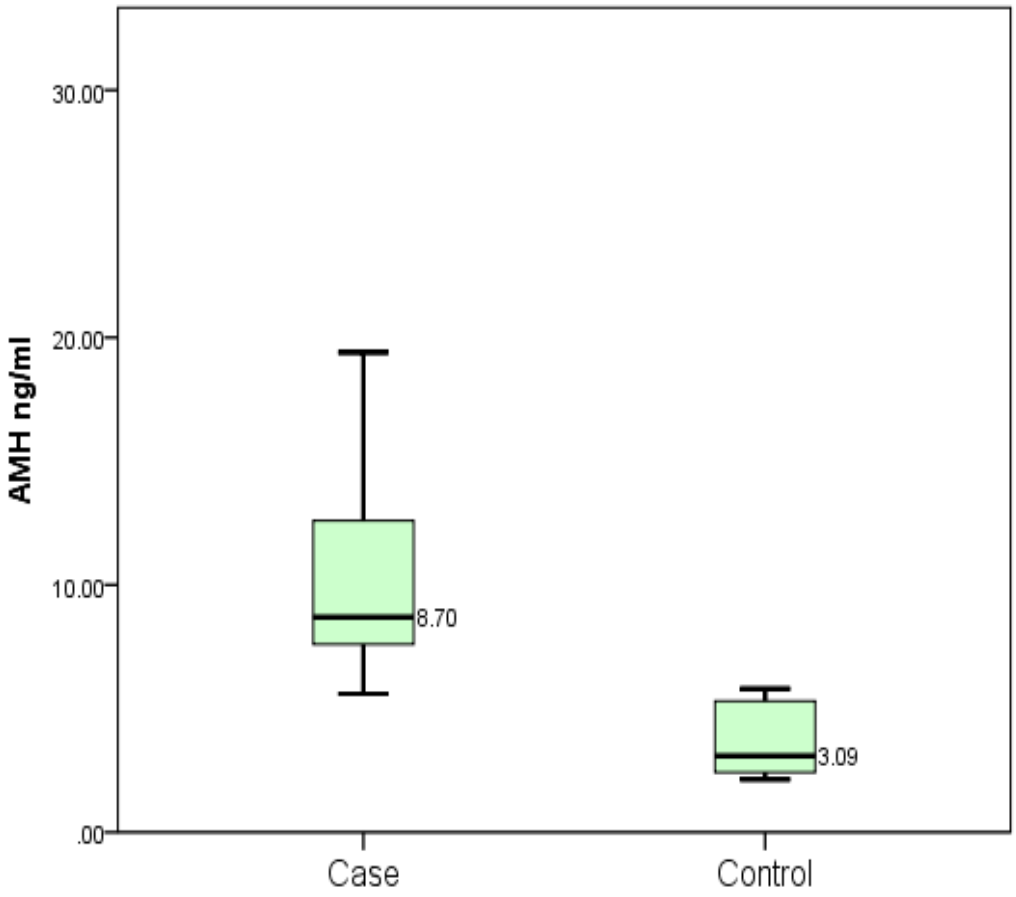


Figure 7: Box plot comparison of anti-mullerian hormone levels (ng/ml) among cases and controls (n=60)

Correlation between AMH levels(ng/ml) and Age

Group	Pearson correlation	p value
Cases (30)	-0.270	0.149
Controls (30)	-0.536	0.002*

**statistically significant at 0.05 level*

In subjects with PCOS, AMH levels had a negative relationship with age but there was no correlation and was not statistically significant while AMH levels in control subjects had a negative linear relationship was statistically significant with age. i,e. As age increases in control subjects, AMH level tend to decrease.

Table 12 Correlation between AMH levels(ng/ml) and Age distribution among cases and controls (n=60)

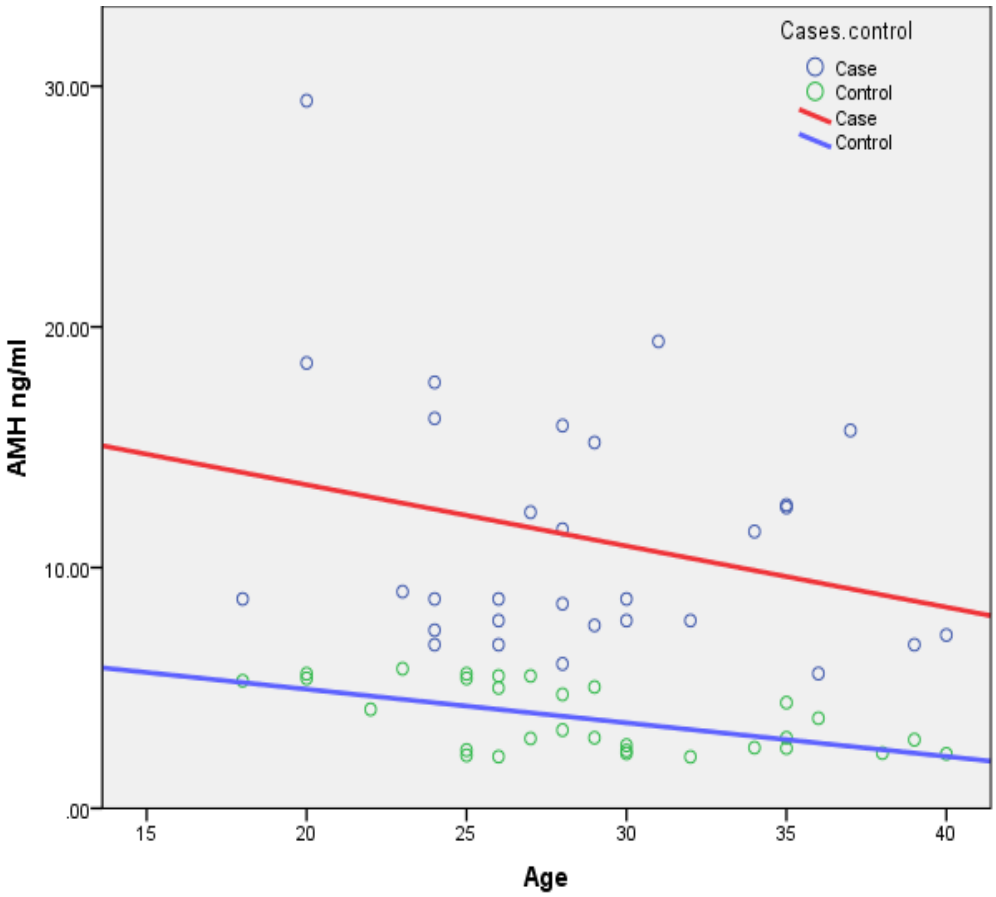


Figure 8: Scatter plot comparison of anti-mullerian hormone levels and age among cases and controls (n=60)

Correlation between AMH levels (ng/ml) and LH (mIU/ml levels

Group	Pearson correlation	*p* value
Cases (30)	-0.335	0.071
Controls (30)	0.449	0.013*

**statistically significant at 0.05 level*

In subjects with PCOS, AMH levels had a negative relationship and was not statistically significant with LH levels .AMH levels in control subjects had a positive linear relationship with LH levels and this correlation was statistically significant. i,e. As LH levels increases in control subjects there is a corresponding increase in AMH levels and vice versa.

Table 13: Correlation between AMH levels (ng/ml) and LH (mIU/ml levels among cases and controls (n=60)

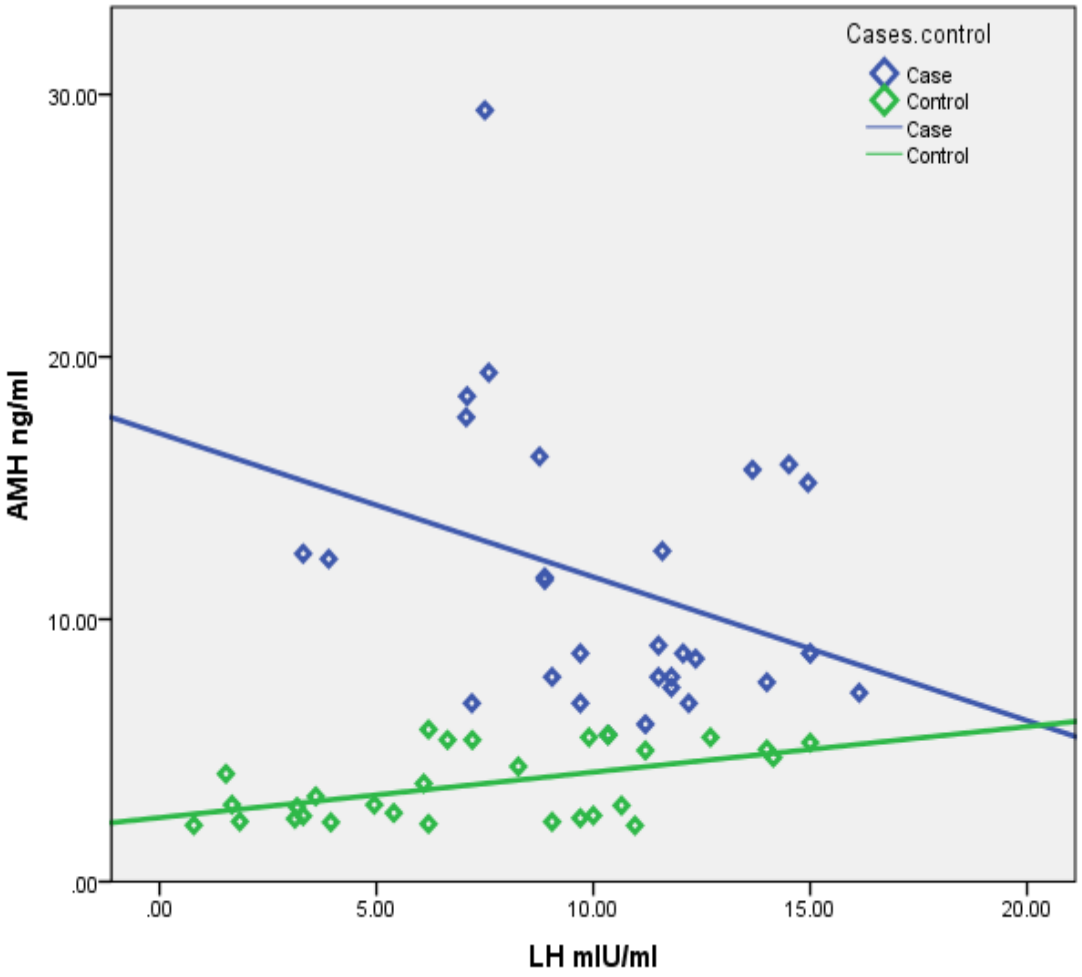


Figure 9: Scatter plot comparison of anti-mullerian hormone levels (ng/ml) and LH levels (mIU/ml) among cases and controls (n=60)

Correlation between AMH (ng/ml) levels and FSH (mIU/ml) levels

Group	Pearson correlation	*p* value
Cases (30)	-0.375	0.041*
Controls (30)	-0.139	0.463

**statistically significant at 0.05 level*

In subjects with PCOS, AMH levels had a negative linear relationship with FSH levels but this correlation was statistically significant while AMH levels in control subjects also had a negative linear relationship with FSH levels but this correlation was not statistically significant. i,e. As FSH levels increases in PCOS subjects there is a corresponding decrease in AMH levels and vice versa.

Table 14 Correlation between AMH (ng/ml) levels and FSH (mIU/ml) levels among cases and controls (n=60)

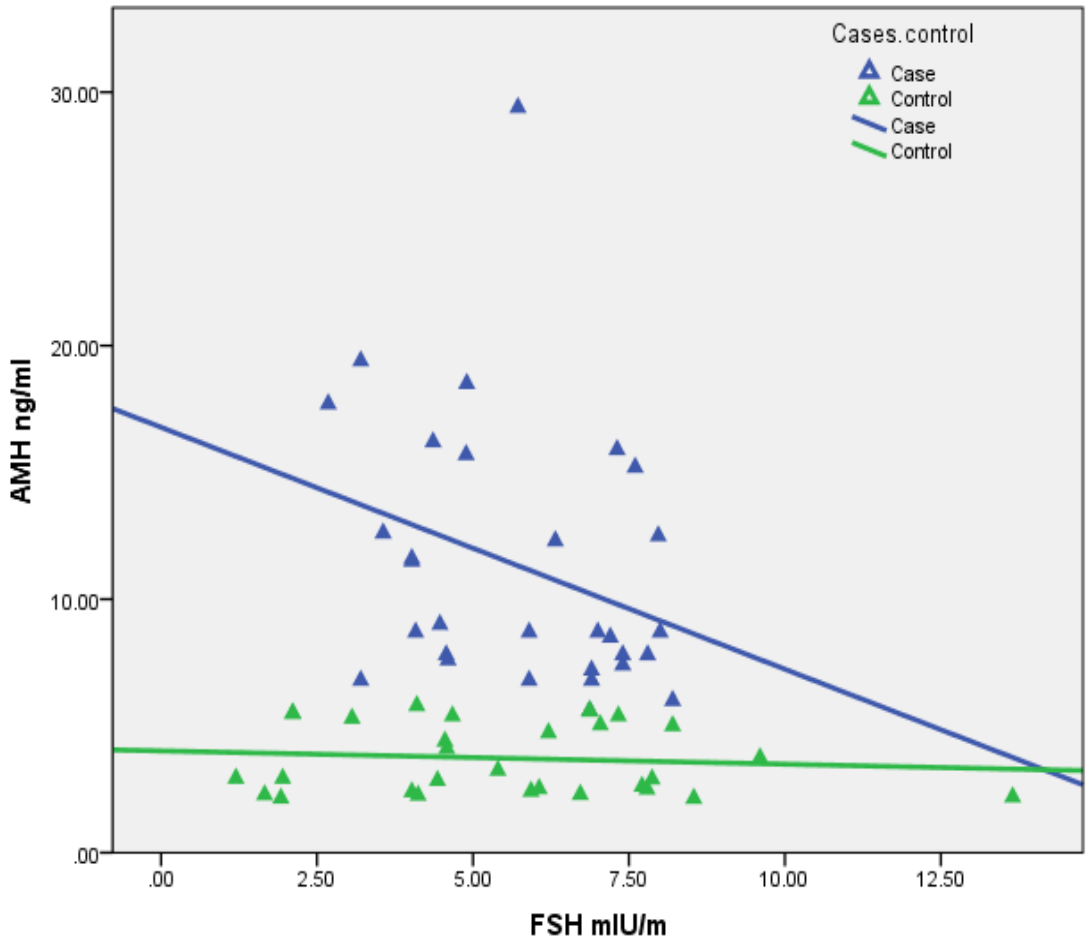


Figure 10: Scatter plot comparison of anti-mullerian hormone levels and FSH levels (mIU/ml) among cases and controls (n=60)

Correlation between AMH levels(ng/ml) and testosterone

Group	Pearson correlation	*p* value
Cases (30)	-0.133	0.485
Controls (30)	-0.328	0.077

In both subjects with PCOS and control subjects, AMH levels had a negative linear relationship with testosterone levels but this correlation was not statistically significant.

Table 15 Correlation between AMH levels(ng/ml) and testosterone levels(ng/ml) among cases and controls (n=60)

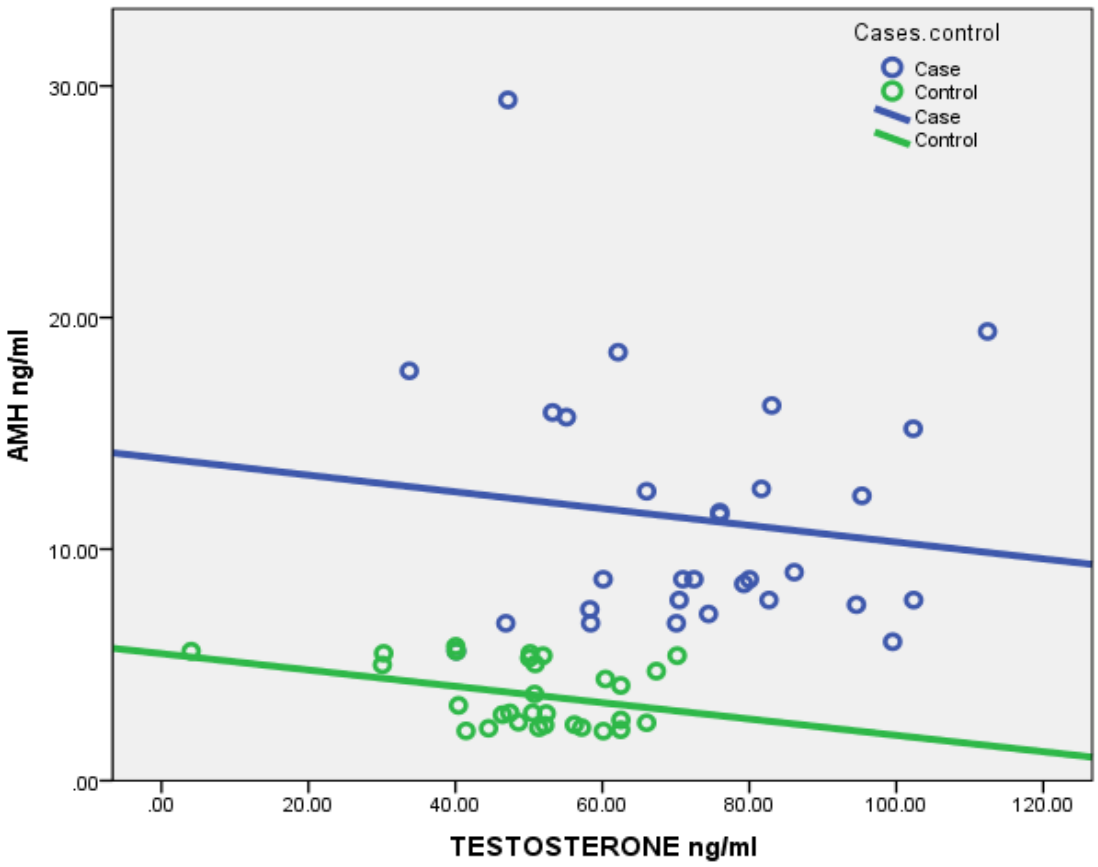


Figure 11: Scatter plot comparison of anti-mullerian hormone levels and testosterone levels among cases and controls (n=60)

Correlation between AMH levels (ng/ml)and combined antral follicular count

Group	Mean combined AFC	Standard deviation	Pearson correlation	*p* value
Cases (30)	30.20	3.662	0.562	0.001 *
Controls (30)	8.20	1.627	0.328	0.077

**statistically significant at 0.05 level*

In subjects with PCOS, AMH levels had a positive linear relationship with combined antral follicular count and this correlation was statistically significant while AMH levels in control subjects also had a positive linear relationship with combined antral follicular count but this correlation was not statistically significant. i,e. As AMH levels are increased in PCOS subjects with high antral follicular count and vice versa.

Table 16 Correlation between AMH levels (ng/ml)and combined antral follicular count in both ovaries among cases and controls (n=60)

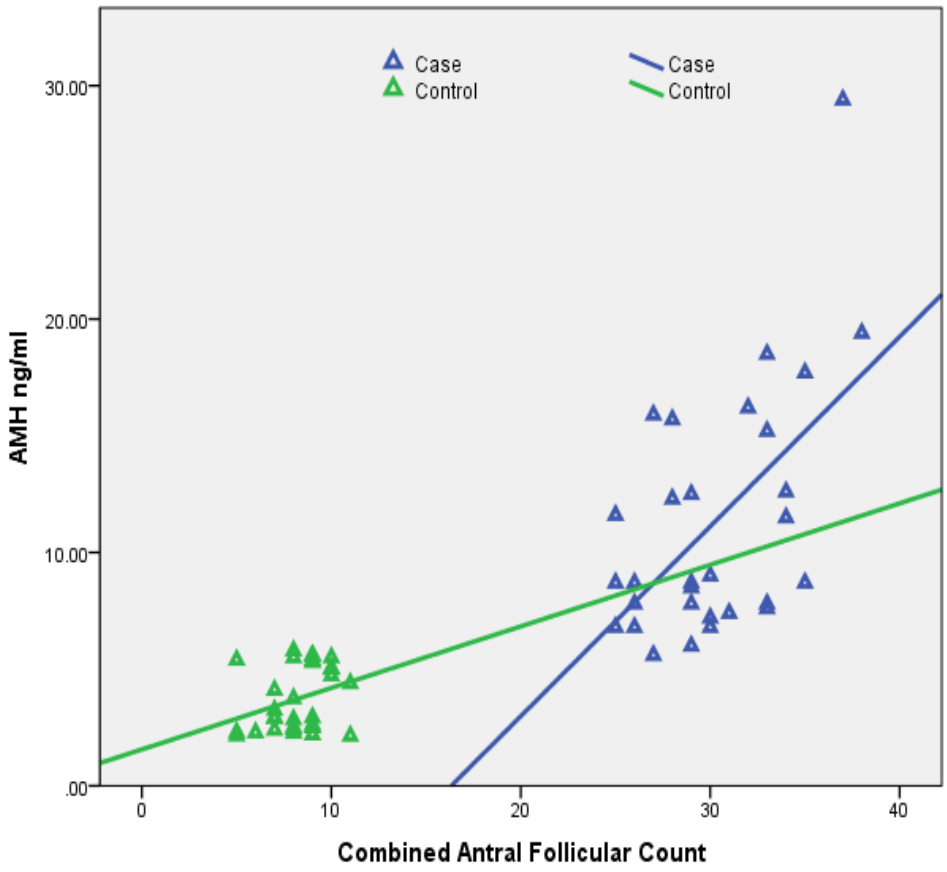


Figure 12: Scatter plot comparison of anti-mullerian hormone levels (ng/ml) and combined antral follicular count among cases and controls (n=60)

Correlation between AMH levels (ng/ml) and combined ovarian volume

Group	Mean combined ovarian volume (cm^3)	Standard deviation	Pearson correlation	*p* value
Cases (30)	30.27	5.34	0.572	0.001 *
Controls (30)	10.28	1.75	0.244	0.193

**statistically significant at 0.05 level*

In subjects with PCOS, AMH levels had a positive linear relationship with combined ovarian volume and this correlation was statistically significant while AMH levels in control subjects also had a positive linear relationship with combined ovarian volume but this correlation was not statistically significant. i,e. As AMH levels are increased in PCOS subjects with high combined ovarian volume and vice versa.

Table 17 Correlation between AMH levels (ng/ml) and combined ovarian volume (both ovaries) (cm^3) among cases and controls (n=60)

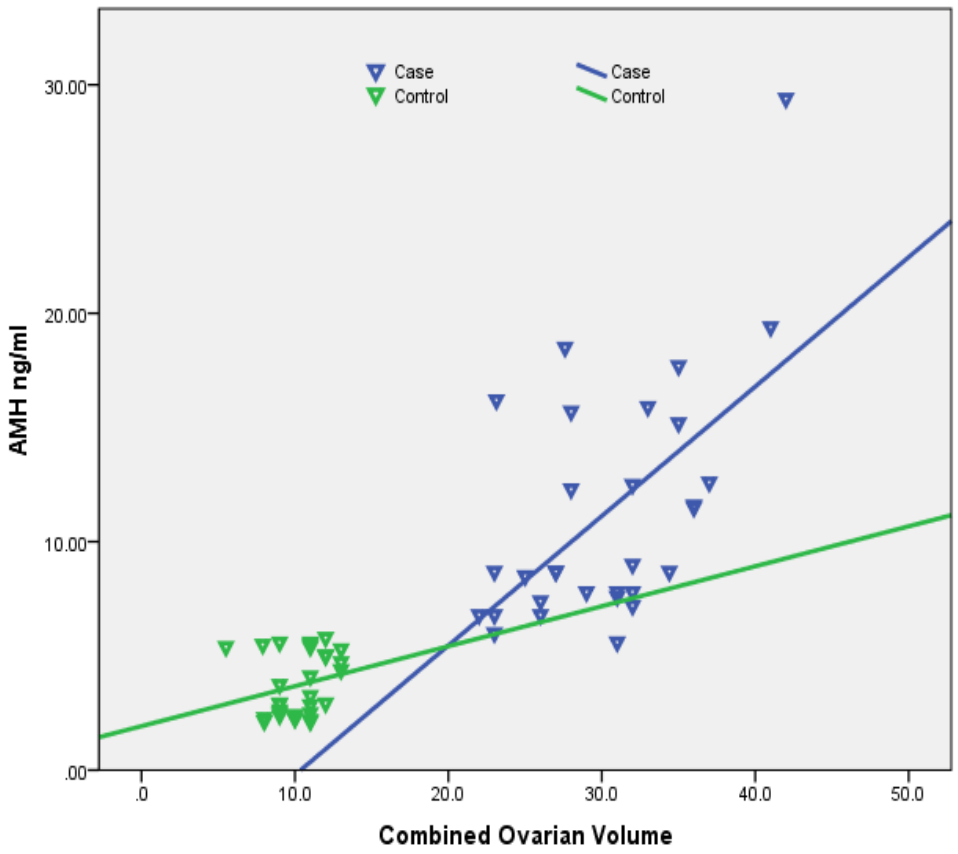


Figure 13: Scatter plot comparison of anti-mullerian hormone levels (ng/ml) and combined ovarian volume among cases and controls (n=60)

Correlation between AMH levels (ng/ml)

and body mass index(Kg/m^2)

Group	Mean BMI (Kg/ m^2)	Standard deviation	Pearson correlation	*p* value
Cases (30)	28.46	478	0.080	0.674
Controls (30)	25.52	5.51	-0.018	0.925

In subjects with PCOS, AMH levels had a positive linear relationship with BMI but this correlation was not statistically significant while AMH levels in control subjects had a negative linear relationship with BMI but this correlation was also not statistically significant.

Table 18: Correlation between AMH levels (ng/ml)and body mass index(Kg/m^2) among cases and controls (n=60)

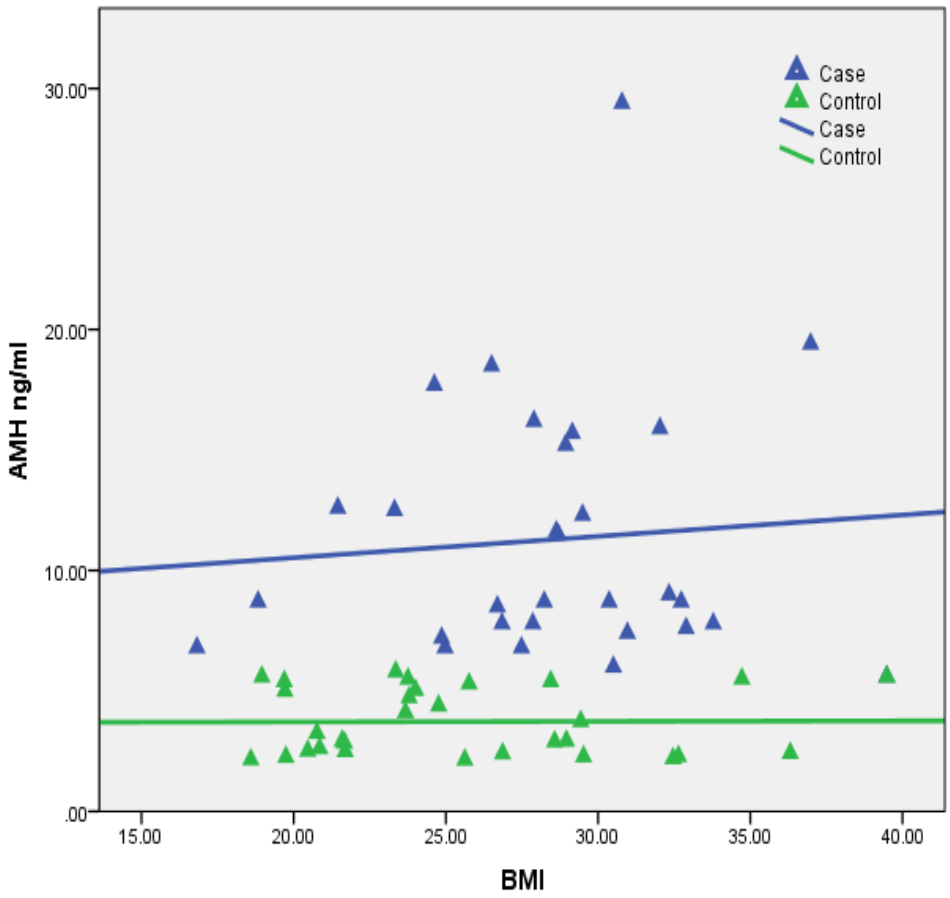


Figure 14: Scatter plot comparison of anti-mullerian hormone levels and body mass index kg /m^2 among cases and controls (n=60)

Correlation between AMH levels (ng/ml) and E_2 (pg/ml) levels

Group	Mean E_2 levels (pg/ml)	Standard deviation	Pearson correlation	*p* value
Cases (30)	71.18	10.88	-0.119	0.531
Controls (30)	73.70	12.50	0.039	0.838

In subjects with PCOS, AMH levels had a negative linear relationship with E_2 levels but this correlation was not statistically significant while AMH levels in control subjects had a positive linear relationship with E_2 levels but this correlation was also not statistically significant.

Table 19: Correlation between AMH levels (ng/ml) and E_2 (pg/ml) levels among cases and controls (n=60)

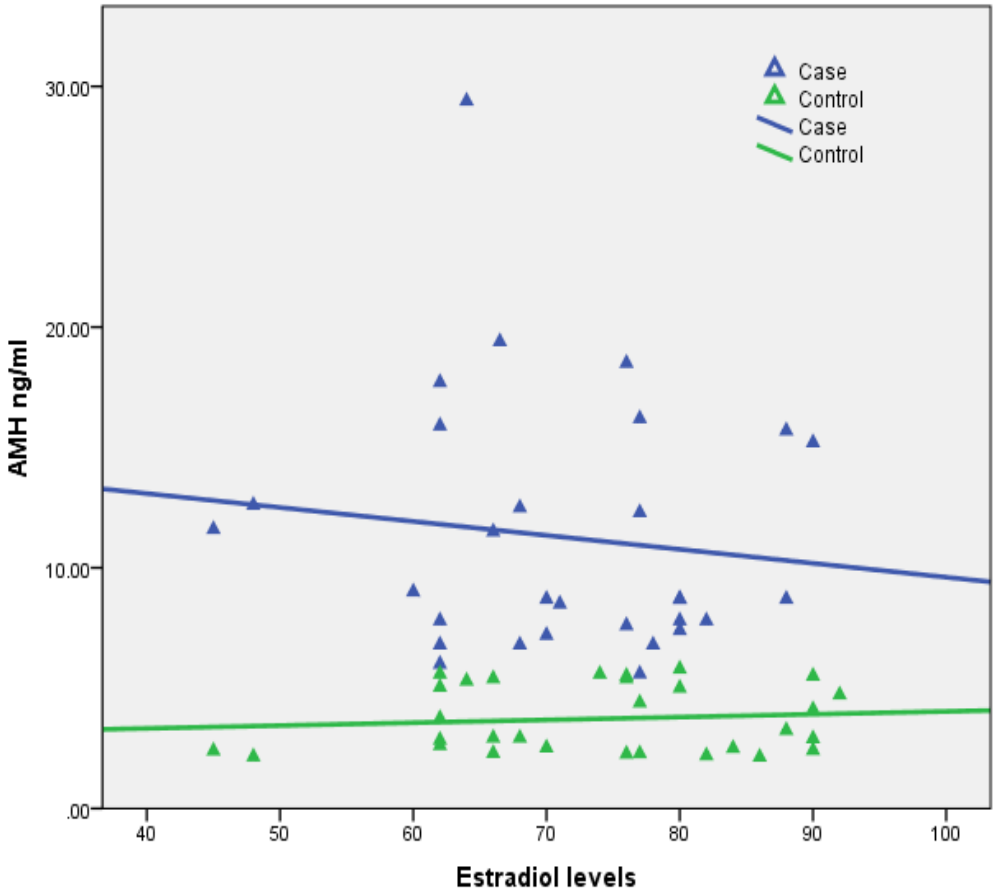
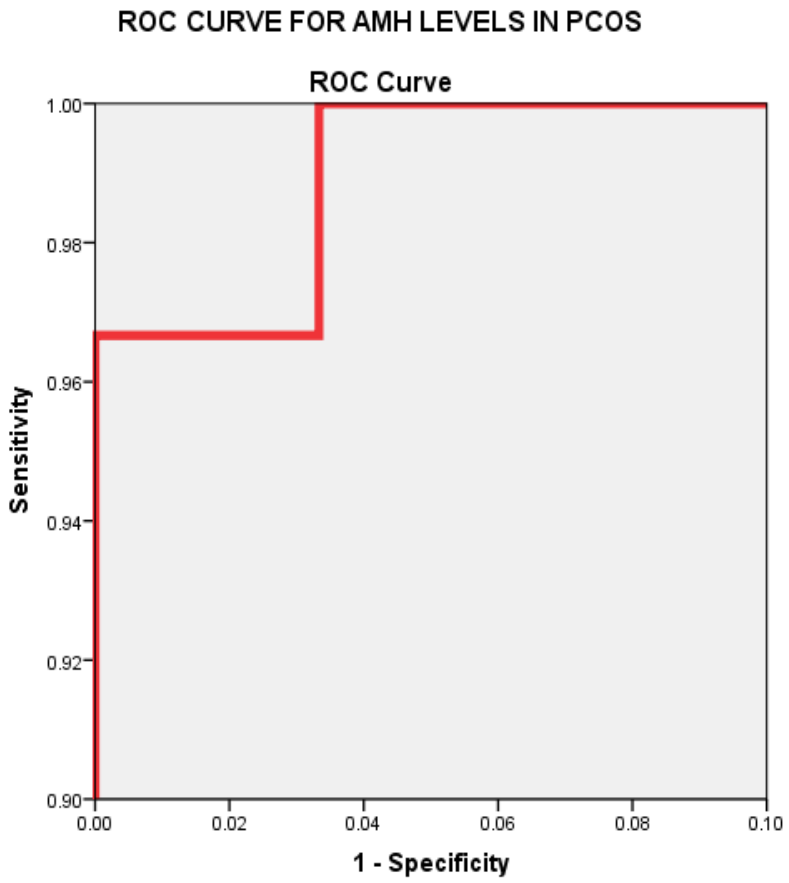


Figure 15: Scatter plot comparison of anti-mullerian hormone levels (ng/ml) and E_2 levels (pg/ml) among cases and controls (n=60)

Figure 16: showing ROC curve of AMH levels (ng/ml) in PCOS (n=60)



Area under the curve (AUC): 0.999

p value for AUC : <0.001* (significant at 0.05 level)

ROC data table for cut-off of AMH levels

PCOS Positive if AMH level ≥ (ng/ml)	Sensitivity	1 - Specificity
1.52 to 5.545	1.000	0.967 to 0.100
5.595	1.000	0.033
5.70	0.967	0.033
5.90	*0.967*	*0.000*
6.40	0.933	0.000
7.00	0.833	0.000

Table 20: ROC data table for cut-off of AMH levels (ng/ml) in PCOS (n=60)

Logistic regression of various factors with PCOS

INDEPENDENT VARIABLE (N)	Odds Ratio (95% CI) for PCOS	*p* value
Age in years (continuous)	0.992 (0.906 – 1.085)	0.854
LH (mIU/ml)	1.262 (1.080 – 1.475)	***0.003***
FSH (mIU/ml)	1.010 (0.817 to 1.250)	0.923
Testosterone levels(ng/ml)	1.102 (1.046 to 1.161)	***<0.001***
AMH levels(ng/ml)	485.11 (241.32 to 756.40)	***<0.001***

Table 21 Logistic regression of various factors with PCOS (n=60)

Serum AMH cut-off level in diagnosing PCOS

1. If we fix AMH level of 5.595 (~6) as a cut-off, sensitivity was 100% in diagnosing all cases of PCOS but 3.3% cases reported false positivity.
2. If we fix AMH level of 5.7 as a cut-off, sensitivity was 96.7% in diagnosing cases of PCOS but 3.3% cases reported false positivity.
3. If we fix AMH level of 5.9 as a cut-off, sensitivity was 96.7% in diagnosing cases of PCOS with a specificity of 100% (no false positives).
4. If we fix AMH level of 6.4 as a cut-off, sensitivity was 93.3% in diagnosing cases of PCOS with a specificity of 100% (no false positives).
5. Based on the current study findings and ROC data table, the cut-off for AMH level between 5.59 to 5.9 units seems appropriate in delineating PCOS subjects and control subjects as it has high level of sensitivity and specificity.

Logistic regression of various factors with PCOS shows that

1. From the above table it is clear that the variable which has maximum influence on the occurrence of PCOS is the serum anti-mullerian hormone levels.
2. The odds ratio for age and FSH levels were not significant and hence cannot be accounted as an association with the occurrence of PCOS
3. Elevated serum levels of LH, testosterone and AMH were found statistically significant predictors of PCOS, with AMH levels demonstrating high odds for developing the diseas

Data analysis

Data were computer analyzed using SPSS/ PC (Statistical Package for the Social Science Inc. Chicago, Illinois USA, version 13.0) statistical package.Simple distribution of the study variables and cross tabulation were applied.The independent-sample t-test procedure was used to compare means of quantitative variables by dividing cases into two qualitative groups such as the relationship between patient and controls hormones.

The results in all the above mentioned procedures were accepted as statistically significant when the p-value was less than 5% ($p<0.05$).

DISCUSSION

Our present study shows that serum AMH levels were low and at the mean value of 3.65 ±1.46 in Normoovulatory women. This study shows serum AMH was higher with a mean value of 11.28±5.29 in Anovulatory PCOS women. Our study shows statistical evidence that the mean AMH levels in controls decrease with increasing age. In PCOS Serum AMH levels increased 2- to 3-fold higher than in control women.

Beyond the age of 38 years the control women had very low serum AMH levels than with women with PCOS . AMH levels were not statistically significant with age and showed a negative relationship. As age increases in control subjects, AMH level tend to decrease

The above inference is supported by studies of Terhi Piltonen.et.al[68].

In the present study the statistical evidence shows that the mean AMH value is not having any significant correlation with LH in control subjects .In subjects with PCOS, AMH levels had a negative relationship and was not statistically significant with LH levels. This is due to the small sample size taken up in the study.

This evidence is supported by the study Artemis Karkanaki.et.al[69].The Control subjects also had a negative linear relationship with FSH levels and this correlation was not statistically significant .In subjects with PCOS, AMH levels had a negative linear relationship with FSH levels and this correlation was found to be statistically significant with AMH levels.

The above correlation is supported by the study A.La.Marca .et.al[70].

AMH levels in control subjects also had a positive linear relationship with combined antral follicular count but this correlation was not statistically significant. In subjects with PCOS, AMH levels had a positive linear relationship with combined antral follicular count and this correlation was statistically significant.

This is supported by the study.D.Dewailly .et.al[71].

AMH levels in control subjects also had a positive linear relationship with combined ovarian volume but this correlation was not statistically significant. In subjects with PCOS, AMH levels had a positive linear relationship with combined ovarian volume and this correlation was statistically significant .While AMH levels in control subjects had a positive and a linear relationship with E_2 levels but this was also not statistically significant In subjects with PCOS, AMH levels had a negative linear relationship with E_2 levels but this was also not statistically significant

This is supported by the study Maxwell Omabe .et.al[72].

In both subjects with PCOS and control subjects, AMH levels had a negative linear relationship with testosterone levels but this correlation was not statistically significant. AMH levels in control subjects had a negative linear relationship with BMI but this correlation was also not statistically significant .In subjects with PCOS, AMH levels had a positive linear relationship with BMI but this correlation was not statistically significant.

The AMH ROC shows that the area under the curve represents the probability that the AMH level for a randomly chosen positive (PCOS) case will exceed the result

for a randomly chosen negative (control) case. Hence the AMH levels of a PCOS subject have a probability of >99% to be higher than AMH levels of a control subject. Therefore it can be said that AMH assay has a very high validity in PCOS.

CONCLUSION

AMH IN NORMOOVULATORY AND PCOS WOMEN

In PCOS there was a marked increase in the serum AMH levels which is of great diagnostic value.Anovulation in PCOS is where the AMH appears to have a major inhibitory role by interfering with during folliculogenesis AMH may be of value in differential diagnosis of oligomenorrhea and it reflects the future of revision of the criteria for the diagnosis of PCOS.

Measurement of AMH can provide a high specificity and sensitivity (92% and 67% respectively) by which it can act as a marker for PCOS. In situations where accurate ultrasonography data are not available this based on the above findings it can be proposed that, in the diagnostic criterion for PCOS than the follicular count.

This can aid in the recoiling the Rotterdam consensus and other definitions for PCOS, especially in women having the Rotterdam PCOS phenotype without HA. We thus propose a simple strategy for the diagnosis of PCOS in clinical practice.

ANNEXURE

MASTER SHEET

STUDY CASES

S:NO	Age	LH mIU/ml	FSH mIU/m	T3 pg/ml	T4 ng/ml	TSH µIU/ml	TESTO ng/ml	AMH ng/ml
1	28	8.88	4.02	1.9	1.2	1.8	75.98	11.6
2	22	14.51	7.31	2.7	1.2	2.4	53.22	15.9
3	20	7.5	5.72	2.76	1.06	3.84	47.15	29.4
4	29	14.95	7.6	2.51	1.2	2.97	102.3	15.2
5	31	7.59	3.2	3.6	1.3	2.9	112.42	19.4
6	20	7.09	5.5.	3.33	0.6	2.9	62.14	18.5
7	21	13.67	4.89	3.45	0.9	4.6	55.11	15.7
8	24	8.76	4.36	3.15	1. 36	1.77	83.04	16.2
9	35	3.31	7.97	3.15	1.2	1.4	66.02	12.5
10	25	3.9	6.32	1.6	0.9	1.5	95.32	12.3
11	24	7.07	2.68	2.98	1. 13	4.5	33.73	17.7
12	26	12.07	4.08	3.15	1.03	4	60.11	8.7
13	25	12.36	7.2	1.38	2.2	4.7	79.25	8.5
14	34	8.88	4.02	2.4	1.5	1.9	75.98	11.5
15	35	11.59	3.56	2.21	2.3	1.4	81.65	12.6
16	23	7.2	3.2	1.24	0.9	4.1	70.07	6.8
17	24	9.7	5.9	1.54	1.6	4.1	80.02	8.7
18	21	10.34	6.87	1.2	1.2	3.6	40.12	5.6
19	18	15	7	1.08	1.2	3	72.46	8.7
20	24	12.2	6.9	1.14	1.5	3.7	46.86	6.8
21	26	11.8	7.4	1.12	1.1	3.7	70.46	7.8
22	23	11.5	4.47	1.24	1.5	2.2	86.12	9
23	25	11.2	8.2	1.14	0.9	1.4	99.51	6
24	29	14	4.6	1.24	1	0.8	94.58	7.6
25	24	16.13	6.9	1.8	1.2	2.3	74.45	7.2
26	22	9.05	7.8	1.42	1.3	3.5	82.67	7.8
27	30	15	8	1.75	1.4	4.1	70.95	8.7
28	23	9.7	5.9	1.66	1.1	3.1	58.41	6.8
29	14	11.8	7.4	1.5	1.5	3.5	58.29	7.4
30	30	11.5	4.57	2.7	1.6	3.6	102.36	7.8

STUDY CASE

PROLACTIN ng/ml	AFC Rt ovary	AFC Lf ovary	Rt Ovary volume cm^3	Lt Ovary volume cm^3	BMI Kg/m^2	E2 pg/ml
6.47	13	12	16	20	28.62	45
10.46	12	15	17	16	32.03	62
12.54	18	19	20	22	30.78	64
7.68	17	16	18	17	28.93	90
6.34	20	18	22	19	36.98	66.5
8.97	17	16	17.6	10	26.5	76
11.28	12	16	11	17	29.15	88
23.57	14	18	7.2	15.93	27.89	77
8.88	12	17	13	19	23.31	68
9.99	12	16	12	16	29.49	77
11.11	17	18	16	19	24.62	62
3.09	17	18	17	17.4	32.73	70
8.36	12	17	13	12	26.69	71
6.47	17	17	17	19	28.69	66
13.52	16	18	18	19	21.45	48
6.04	14	12	10	12	16.82	62
08-Jan	13	12	13	10	28.23	88
6.84	15	12	17	14	39.48	77
6.04	14	12	15	12	30.36	80
10.1	16	14	11	15	27.48	68
11.1	12	14	13	16	27.85	62
12.8	12	18	13	19	32.33	60
12.3	17	12	13	10	30.5	62
6.8	15	18	12	19	32.89	76
10.16	12	18	11	21	24.86	70
7.64	18	15	11	20	26.84	80
10.75	13	16	13	14	18.83	80
11.4	17	8	13	10	24.97	78
6.04	13	18	15	11	30.96	80
8.9	14	15	15	17	33.78	82

STUDY CONTROL

S:NO	Age	LH mIU/ml	FSH mIU/m	T3 pg/ml	T4 ng/ml	TSH µIU/ml	F.TESTO ng/ml	AMH ng/ml
1	27	9.9	2.11	1.63	1.7	5.4	30.23	5.5
2	21	10.34	6.87	1.52	8	3.6	40.12	5.59
3	26	11.2	8.2	3.1	1.7	0.19	30.07	5
4	29	14	7.04	1.96	1.8	2.04	50.87	5.04
5	18	15	3.06	1.74	1.2	4.05	50.08	5.3
6	23	6.2	4.1	1.98	1.4	4	40.05	5.8
7	27	10.65	7.87	3.21	1.15	5.12	52.36	0.9
8	28	14.15	6.21	3.1	1.05	5.21	67.35	4.73
9	21	7.21	7.33	3.46	1.16	2.27	70.18	5.4
10	35	8.27	4.55	2.34	1.11	1.32	60.42	4.39
11	35	3.31	7.79	3.15	1.21	1.39	66.02	2.5
12	36	6.09	9.6	3.67	1.55	3.91	50.77	3.74
13	25	6.64	4.67	3.41	1.03	2.13	51.91	5.4
14	26	12.7	2.11	2.4	1.4	2	50.17	5.5
15	32	10.96	8.54	1.7	1.08	2.9	60.13	2.14
16	22	1.53	4.58	2.7	1.3	2.9	62.5	4.11
17	25	6.2	13.65	2.7	1.3	2.9	62.5	2.2
18	40	3.95	4.12	1.7	1.5	1.4	44.51	2.26
19	30	5.4	7.71	1.9	1.2	2.2	62.51	2.62
20	25	9.7	5.93	2.1	1.1	0.9	56.19	2.42
21	38	1.85	6.72	2.6	1.3	2.4	57.18	2.29
22	30	3.12	4.02	1.8	1.2	2.1	52.18	2.4
23	30	9.05	1.66	2.1	1.4	1.5	51.35	2.28
24	25	10.34	6.87	2.6	1.4	1.2	4.04	5.59
25	34	10	6.06	1.8	1.2	1.3	48.58	2.52
26	39	3.17	4.43	2.2	1.1	1.7	46.4	2.85
27	26	0.79	1.92	2.3	1.5	1.7	41.45	2.15
28	29	4.95	1.95	2	1.7	2.1	50.5	2.93
29	35	1.67	1.2	2.2	1.2	1.4	47.41	2.93
30	28	3.6	5.4	2.1	1.4	1.8	40.42	3.25

STUDY CONTROL

PROLACTIN ng/ml	AFC Rt ovary	AFC Lf ovary	Rt volume cm^3	Lf volume cm3	BMI Kg/m^2	E2 pg/ml
20.17	5	5	5	6	34.72	76
3.1	5	4	5	4	39.48	74
21	4	6	5	7	19.71	80
24	6	4	7	5	24	62
9.12	4	5	6	7	25.76	64
10.31	3	5	5	7	23.35	80
15.2	4	3	5	4	28.57	90
14.74	6	4	7	6	23.79	92
12.31	4	5	6	5	19.69	66
8.01	5	6	6	7	24.76	77
8.88	4	5	5	6	21.69	84
9.84	3	5	4	5	29.43	62
10.26	3	2	3	2.5	28.44	76
20.14	4	4	3.9	4	23.75	90
8.02	6	5	7	4	25.62	86
8.02	4	3	5	6	23.67	90
8.02	5	4	6	5	32.46	82
10.24	3	5	5	5	19.74	76
20.01	4	5	4	5	20.85	62
8.48	3	5	4	6	36.31	90
9.02	2	3	3	5	32.64	66
10.74	3	4	4	5	26.86	45
12.12	2	4	5	5	29.52	77
15.26	4	5	5	6	18.95	62
14.01	3	5	3	6	20.46	70
15.12	4	4	5	6	21.67	62
8.24	3	2	4	4	18.59	48
8.17	4	5	5	7	21.59	66
23.52	3	4	4	5	28.96	68
11.31	3	4	5	6	20.76	88

CASE PROFOMA

"A STUDY OF RELATIONSHIP OF ANTIMULLERIAN HORMONE IN WOMEN WITH NORMOOVULATORY AND POLY CYSTIC OVARIAN SYNDROME"

Name: **IP/OP.NO:**

Age/Sex:

Place:

Socioeconomic status: Low/Middle/High

Chief C/O:

Ovulatory /Anovulatory-

Infertility-

Menstural H/O:

Duration-

Last Menstural Periods-

Marital H/O:

Duration-

Consagunity-

Obstertrics H/O:

Gravida- Para- Living- Abortion-

Last Child Birth-

Past H/O:

DM/HT/Allergy/Asthma/Treatment/Surgery

Family H/O:

Personal H/O:

General examination:

Ht: Wt: BMI:

RR: PR: B.P:

Thyroid:

Hirsutism:

Pattern of hair distribution	score
• Upper lip	
• Chin	
• Chest	
• Upper back	
• Lower back	
• Upper abdomen	
• Lower abdomen	
• Upper arms	
• Forearms	
• Thighs	
• Legs	

Systemic examination:

CVS:

RS:

Abdomen:

CNS:

Investigations:

CBC:

FBG:

LH	
FSH	
fT3	
fT4	
TSH	
PRL	
T Testosterone	
E2	
AMH	

Ultrasonography:

Uterus		
Ovarian volume (OV)	Rt. Ovary (cm^3)	Lf. Ovary (Cm^3)
Antral follicular count (AFC)	Rt. Ovary	Lf. Ovary

Semen analysis:

Diagnosis:

Signature of the principle investigator:

Consent Form

You are requested to participate in a study conducted in the Department of Biochemistry, Chennai Medical College Hospital & Research Centre, Irungalur, Trichy, Tamilnadu titled "A study of Relationship of Antimullerian Hormone in women with Normoovulatory and Poly Cystic Ovarian Syndrome". Your participation in the study is voluntary

- There will be no cost for participating in the study
- Your participation is not a compulsion
- You have the right to withdraw from the study at any time.

Nature of Study:

- ✓ If any abnormalities are identified, you will be informed for further consultation.
- ✓ The results of this study will be kept confidential

We believe that the results of this study will be beneficial for advancements in medicine & Science. We assure you that we will not use these result for any other purpose.

Consent

I Mr /Mrs / Ms_____ residing at _____ _____on this day _____ after having read the consent form carrying information for the above mentioned study and I hereby give my consent to take 5ml of my blood sample for the purpose of doing diagnostic tests including hematological , biochemical investigations. I was explained about the procedure in detail and give my consent for participating in the study and for using the results for Medical & Scientific purposes.

Signature of the participant

Signature of the Investigator

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